

ABSTRACT

Title of Dissertation / Thesis: A MODEL TO PREDICT FLUCTUATIONS IN
RUMEN pH

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Factors affecting pH and the mechanism of rumen pH control are poorly understood. Meta-analysis was conducted to estimate the effect of sodium bicarbonate (NaHCO_3) on rumen pH. Addition of NaHCO_3 increased strong ion difference (SID) and rumen pH while, volatile fatty acid concentration remained unaffected. Single-compartment model is proposed to predict the changes in rumen pH when NaHCO_3 is added to diet. Prediction of model was acceptable and there were no significant mean or linear biases. An in-vitro study was conducted to determine uptake of macro-minerals by rumen microbes and the changes in SID. Differences were found in microbial mineral composition due to different pellet (liquid and solid-associated bacteria), buffer strength, pH, feed (alfalfa hay, corn grain) and length of incubation (4, 14, or 24 h). On average microbes took up more cations than anions. The values obtained from these experiments can be used to predict changes in rumen SID.

A MODEL TO PREDICT FLUCTUATIONS IN RUMEN pH

By

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List of Abbreviations

Δ [SID]	change in concentration of strong ion difference
$\Delta[\text{Na}^+]_r$	change in amount of Na^+ in rumen
0.5x	half times the concentration of Van Soest buffer at pH6.8
1x	normal concentration of Van Soest buffer at pH 6.8
1x, pH 5.8	normal concentration of Van Soest buffer at pH 5.8
2x	two times the concentration of Van Soest buffer at pH 6.8
Ca^{2+}	calcium ion
Cl^-	chloride ion
Dietary Na^+	quantity of Na^+ in diet
DM	dry matter
DMI	dry matter intake
HCO_3^-	bicarbonate ion
K^+	potassium
k_a	rate of absorption
k_d	fractional out-flow rate
k_p	rate of passage
LAB	liquid associated bacteria
Mg^{2+}	magnesium ion
M	moles per liter
mol	moles

Na^+	sodium ion
NaHCO_3	sodium bicarbonate
OM	organic matter
OMF	organic matter fermented
PO_4^{2-}	phosphorus
pCO_2	partial pressure of CO_2
r^2	simple coefficient of determination
RMSPE	root mean square prediction error
S^-	sulfide ion
SAB	solid associated bacteria
SEM	standard error of means
SID	strong ion difference
VFA	volatile fatty acids
VFA^0	un-dissociated volatile fatty acid

Chapter 1: REVIEW OF LITERATURE

Introduction

Low rumen pH depresses feed intake and fiber digestion, and may lead to metabolic disorders (Rogers et al., 1982). Rumen pH is a function of rates of production and absorption of volatile fatty acids (VFA) by microbes (Rumsey et al., 1970), water flux across the rumen wall, saliva flow and its constituent buffer flow into the rumen, feed acidity, and digesta passage to the lower gastro-intestinal tract (Bailey et al., 1961; Baldwin et al., 1987; Murphy et al., 1982; Rumsey et al., 1970; Sutton, 1985). Hence, there should be a balance between all these factors, to maintain the rumen pH within a normal range. Low rumen pH may also decrease dry matter intake (DMI), microbial yield (Mould and Ørskov, 1983) and milk composition (Gaynor et al., 1994). Therefore, diets should be formulated to maintain rumen pH within the optimal range.

Current feeding practices involve feeding buffers to cattle fed high-concentrate diets to ameliorate changes in rumen pH. However, factors affecting rumen pH and the mechanisms of rumen pH control are poorly understood. Effect of buffers on VFA production and concentration in the rumen, and effect of the rate of removal of sodium ions (Na^+) and potassium ions (K^+) from the rumen have not been established. Previous literature suggests that varying the proportions of concentrate to forage affects VFA concentration and rumen pH. A few studies showed that addition of buffers decreased VFA concentration (Erdman et al., 1982; Staples et al., 1989), while other studies showed no effect of buffers on rumen VFA concentration

(Snyder et al., 1983; Stokes et al., 1986; Ghorbani et al., 1989; Kennelly et al., 1999 etc.). The question of differential effect of buffers on these parameters in diets varying in amount of concentrate remains unanswered.

Stewart (1983) stated that the concentration of hydrogen ions (H^+) and bicarbonate ion (HCO_3^-) of biological solutions is regulated by three independent variables: partial pressure of CO_2 (pCO_2), difference in concentration of strong cations and anions (**SID**), and concentration of partially dissociated weak acids at all times. Kohn, (2000) adapted Stewart's SID theory for rumen pH regulation and stated that there are three independent variables that regulate the rumen pH: 1) SID, 2) pCO_2 , and 3) partially dissociated weak acids ($[\text{VFA}] = \text{un-dissociated } [\text{VFA}] + [\text{VFA}^-]$). This understanding provides the basis for a mathematical model to predict rumen SID and pH for different diets and feeding conditions.

Another aspect for predicting rumen SID is estimation of the uptake of minerals by microbes. Microbes attach to feed and absorb nutrients and minerals into their cytoplasm. The major factors that influence the release of nutrients from the diet are: type of diet and its mineral content, pattern of feeding, rumination, salivary volume, composition and flow rate from the rumen (Johnson and Jones, 1989). However, if minerals are taken up by the microbes they are likely to play a major role in regulating the intracellular pH (Krulwich, 1983) and rumen pH by altering SID in the rumen. Moreover, availability of nutrients within feed materials is dictated largely by the physical structure of forage or grain being digested (McAllister et al., 1990) and the concentration of growth medium (Poupard et al., 1973). The question

of mineral uptake by rumen microbes as affected by changes in microbial environment remains unanswered.

Role of Strong Ion Difference in Regulating Rumen pH

Concept of Strong Ion Difference

Strong ion difference is defined as the difference of the sum of concentration of strong cations and strong anions in the solution. It is synonymous to the buffer base described by Singer and Hastings (1948). Stewart (1983) proposed that SID, $p\text{CO}_2$, and total protein (A_{tot}), are the three independent variables which regulate the six primary dependent variables (H^+ , hydroxyl ion (OH^-), protein anions (A^-), bicarbonate ion (HCO_3^-) and carbonate ion (CO_3^{2-})), and at all times maintain their concentration. Strong ion difference theory is based on three fundamental concepts:

- 1) electro-neutrality must be maintained (sum of all positive charges must equal the sum of all negative charges);
- 2) mass must be conserved; and
- 3) dissociation constant of water determines the dissociation of a substance in water.

If Stewart's theory is valid then the movement of H^+ between solutions (by ion channels or pumps) will not affect the local H^+ concentration. Directly adding or removing H^+ to or from one of the compartments will not alter the value of any of the independent variables present and hence H^+ concentration will be maintained at the same value as before by a change in the dissociation of water to reverse any H^+ concentration fluctuations. The dissociation of water equilibrium is able to provide an essentially inexhaustible source or sink for H^+ ions (Sirker et al., 2002). Thus, any change in pH is due to the changes which are produced by independent variables.

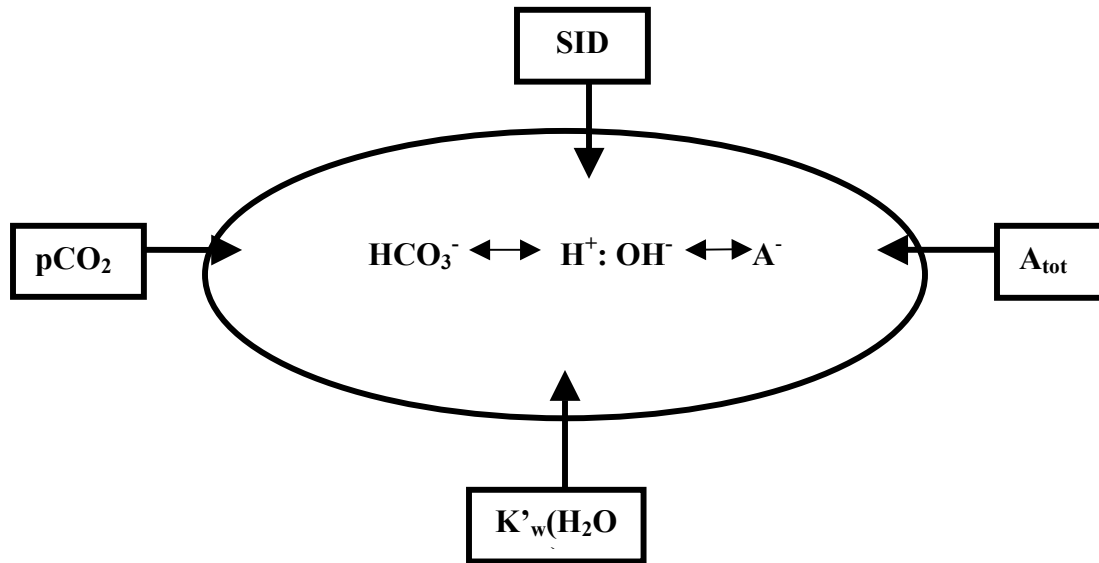


Figure 1.1 The acid-base system. Arrows indicate the influence that variables may exert on each other. Single ended arrows indicate the effect of the independent variables: strong ion difference concentration [SID], partial pressure of CO_2 (pCO_2), and total protein concentration [A_{tot}] on the dependent variables hydrogen ion [H^+], hydroxyl ion [OH^-], bicarbonate ion [HCO_3^-], dissociated protein anions [A^-], and (not shown) carbonate ion concentration [CO_3^{2-}], and effective dissociation constant of water ($\text{K}'_{\text{w}} = \text{H}_2\text{O} \times \text{K}_{\text{w}}$). Dependent variables are enclosed in ellipsis, to indicate that they will all be influenced by a change in any one of the independent variables (adapted from Jones, N.L. 1990).

According to Stewart (1983), the acid-base status of a system can be summarized as:

- 1) the concentration of H^+ , pH, and HCO_3^- of each body fluid is determined by pCO_2 , SID, and total plasma protein concentration [A_{tot}] values in that fluid.
- 2) normally, SID and pCO_2 changes determine the changes in [H^+], pH, and [HCO_3^-] in body fluids as proteins are not freely permeable across the biological membranes.
- 3) changes in pCO_2 are well controlled by removal via expiration and eructation.

4) Strong ions move between body fluids through membranes and the resulting changes in SID values provide the major mechanism for acid base interaction in body fluids.

The major factors affecting SID in the rumen are type of diet and its mineral composition, buffers, uptake of minerals by microbes, salivary mineral composition, mineral dilution rates and absorption of minerals from the rumen wall.

Inflow of Minerals into the Rumen Pool

Feeds vary in buffering capacity depending upon the content of protein, starch, water holding capacity and the intrinsic osmotic pressure of feedstuffs (Sylvie et al., 2000). Mineral composition of the feed may also vary with stage of harvesting, type of soil, processing of feed, and potential of degradation in rumen. After fermentation, nutrients are released from the feed into the rumen pool.

Saliva is a mixed secretion from the parotid, sub-maxillary and sub-lingual glands. The main ions present in saliva are Na^+ , HCO_3^- , phosphate (PO_4^{2-}), chloride (Cl^-), and K^+ . It has been estimated that in cattle 15 to 30 M of Na^+ (Bailey et al., 1961) flow into the reticulo-rumen per day. Factors that affect the rate of flow of saliva include feed dry matter, (Balch, 1958; Erdman, 1988), amount of forage in diet (Balch, 1958; Erdman, 1988) and forage particle size (Rumsey, 1972; Erdman, 1988). Total saliva flow added per unit feed intake by cattle is double the rate for alfalfa hay compared to freshly cut hay (Meyer et al., 1964). Bailey and Balch, (1961) found no change in the amount of saliva secreted after rumen pH was changed from 6.8 to 5.4 by adding acetic acid to rumen contents intra-uminally. Saliva production is related to the time spent chewing; saliva output decreases with decrease in forage particle

size (Woodford et al., 1986) because of the effect of chewing time. All these factors indicate a reduced capacity of the cattle to neutralize rumen acids, via natural buffering action of saliva due to low forage diets (Staples et al., 1989).

Role of Buffers in Regulating Rumen Environment

A buffer can be defined as a salt of a weak acid or oxide or hydroxide which neutralizes the acids present in feeds, or acids produced during nutrient digestion and metabolism (Chalupa and Schneider, 1985). A true buffer should lessen the decrease in pH without causing an increase in pH. A buffer usually consists of a weak acid and its conjugate base (strong base), which are present in equilibrium, or at high pH a weak base and its conjugate acid.



If an acidic solution is added to the system, the conjugate base consumes the H^+ added to form conjugate acid (resisting a large change in pH).

Buffering action of a system depends upon the strength of the buffer and the concentration of acid-base at a particular pH, which in turn depends on the pK_a of the buffer (adapted from Segel, 1976).

$$\text{pK}_a = -\text{Log} (k_a)$$

where k_a (acid dissociation constant) (mol^2/L^2) is the strength of acid in aqueous solution. Thus,

$$k_a = k_{\text{eq}}[\text{H}_2\text{O}],$$

$$\text{where } k_{\text{eq}} = [\text{H}_3\text{O}^+] [\text{A}^-] / [\text{HA}] [\text{H}_2\text{O}].$$

The stronger the acid, the larger is its k_a value, and smaller is its pK_a value (adapted from Miller et al., 1974).

The pK_a of a solution is the pH at which addition of H^+ (M) or OH^- (M) produces the least change in pH. Thus, any acid-base buffer is effective at and around its pK_a (Segel, 1976).

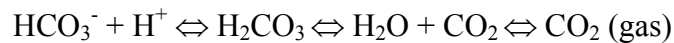
The most common dietary additives currently used in practice as buffering agents are sodium bicarbonate (**NaHCO₃**), potassium bicarbonate (**KHCO₃**), sodium sesquicarbonate, and magnesium oxide. Addition of buffers to low forage rations has been shown to counter milk fat depression in dairy cattle (Davis et al., 1964; Emery et al., 1965; Thomas and Emery 1969; Thomas et al., 1984), and increase the rumen pH (Kilmer et al., 1981; Erdman et al., 1982; Rogers et al., 1982; Okeke et al., 1983; De Peters et al., 1984; Staples et al., 1986; Stokes and Bull, 1986; West et al., 1986) or show no effect (Kilmer et al., 1980; Snyder et al., 1983; Eickelberger et al., 1985; Rogers et al., 1985; Stokes et al., 1986). A study conducted by Bigner et al. (1997) showed that both, sodium propionate and NaHCO₃, have similar buffering capacities indicating that it is the Na^+ that plays an important role in maintaining the ruminal pH.

Addition of NaHCO₃ to the high grain diets may aid in protein utilization in lactating cows (Trenkel, 1979). However, no improvements in nitrogen balance on feeding 1 % NaHCO₃ with corn silage: grain (60: 40) diet has also been reported (Erdman et al., 1982). Rumen ammonia (**NH₃**) concentration increases with an increase in rumen pH on addition of NaHCO₃ (Snyder et al., 1983, Okeke et al., 1983). This effect may be due to increased solubility of protein with increase in pH (Wholt et al., 1973), which may cause higher concentration of NH₃ in the rumen (Snyder et al., 1983).

Addition of buffers increases (Harrison et al., 1975; Owens et al., 1979; Rogers and Davis, 1982), or has no effect (Erdman et al., 1982; Rogers et al., 1982; Stokes et al., 1985; Jacques et al., 1986; Stokes et al., 1986) on liquid dilution rate (**LDR**). Rumen osmolarity increases when 5 % NaHCO₃ is added to a diet consisting of 50 % corn silage. However, infusions of NaHCO₃ at a concentration of 288 g/d increases osmolarity of high roughage diets but not of high concentrate diets (Rogers and Davis, 1982). Snyder et al. (1983) reported no significant effect of NaHCO₃ addition on urine volume and fecal DM output.

Role of Partial Pressure of CO₂ in Regulating Rumen Environment

Applying the concepts of SID theory to the rumen, Kohn and Dunlap (1998) explained the effects of partial pressure of CO₂ on rumen pH. Rumen gases and liquids are in close proximity to maintain equilibrium between soluble and evolved gases in the rumen. The overall equilibrium constant (k_{eq}) for the three reactions (equation) is the product of three equilibrium constants for each of the reactions in which CO₂ gas is converted to HCO₃⁻.



$$k_{eq} = k_1 k_2 / k_{-1} k_{-2} = ([\text{HCO}_3^-] [\text{H}^+]) / ([\text{CO}_2] [\text{H}_2\text{O}])$$

Reorganizing this equation and taking Log₁₀ of both sides provides the Henderson-Hasselbalch equation:

$$\text{pH} = \text{p}k_a + \text{Log} ([\text{HCO}_3^-] / [\text{CO}_2])$$

Moreover, the equilibrium solubility of CO₂ is determined from the partial pressure in atmospheres (**atm**) of CO₂ in the gas phase (pCO₂) and Henry's constant (k). Thus,

$$[\text{CO}_2]_{aq} = k(\text{pCO}_2)$$

where $[\text{CO}_2]_{\text{aq}}$ is the concentration of dissolved CO_2 in solution. Henry's constant (k) for CO_2 in solvent for 0.15 ionic strength at 37 °C is 0.0229 M/atm (adapted from Segel, 1976). Thus, the effective $\text{p}K_a$ ($\text{p}K_a'' = 7.74$) for the three-reaction system is ($\text{p}K_a + \text{p}K_a(\text{CO}_2) = (6.1 + -\text{Log}(0.0229))$). The extent of CO_2 released from the rumen will therefore depend upon the pCO_2 in the rumen as

$$\text{pH} = 7.74 + \log \left(\frac{[\text{HCO}_3^-]}{\text{pCO}_2} \right)$$

The eructation process in the rumen is a unidirectional process, with the gas leaving the rumen when total pressure exceeds atmospheric pressure (Stevens and Sellers, 1960). Hence, rumen gases are not in equilibrium with the outside air.

The pressure of CO_2 depends on the ideal gas law:

$$PV = nRT$$

where P represents pressure (atm), V is the volume (liters), n is the M of gas, R is the gas constant ($0.8206 \text{ L atm K}^{-1} \text{ mol}^{-1}$), and T is the temperature (degrees Kelvin). But under rumen conditions, pressure remains relatively constant as CO_2 is eructated by the animal (Kohn and Dunlap, 1998).

The three-reaction system is important in regulating rumen pH because the concentration of any of the reactants decreases the shift in the reaction towards the reactant. For example if CO_2 is removed, HCO_3^- accepts H^+ to form CO_2 resulting in an increase in rumen fluid pH.

Role of Volatile Fatty Acids in Regulating Rumen Environment

Role of Volatile Fatty Acids in Ruminant Nutrition

Short chain fatty acids are the result of fermentation of organic matter by microbes present in rumen. Acetic, propionic and butyric acids are the predominant

forms of VFA in the rumen. These VFA have a pK_a of ≤ 4.8 (Counotte et al., 1979) whereas the pH of rumen fluid is around neutrality (6.2 to 6.8), therefore majority of VFA are present as anions (VFA^-) rather than in an un-dissociated (VFA^0) form (Bergman, 1990). The type of diet consumed affects the amount and nature of fermentation end products formed (Hungate, 1968; Slayters, 1979; Russell, 1988; Bergman, 1990). The molar concentration of VFA produced varies from 60 to 150 mM in the rumen fluid (Dijkstra et al., 1993). The concentration of VFA (M) in rumen peaks at 2 to 4 h post-feeding but, high forage diets produce lesser fluctuation in VFA concentrations throughout the day (Dijkstra et al., 1993).

High grain diets are rapidly fermented, enhancing the growth of propionate and lactate-producing organisms in the rumen (Bergman, 1990). The increase in concentration of propionate has a negative effect on milk fat, producing low milk fat syndrome (Lindsay and Pethwick, 1983). Use of antibiotics such as monensin favors propionate production while the production of acetate, butyrate and precursor of methane are reduced (Chen and Wulin, 1984; Richardson et al., 1976; Wolin and Miller, 1983). Thus, concentration of total and individual VFA in the rumen is highly variable and depends on the time after feeding and on the composition of diet.

Production and Absorption of Volatile Fatty Acids

VFAs produced in the rumen are either absorbed through the rumen wall or passed to the lower tract (Dijkstra, 1993). Carter and Grovum, (1990) proposed a mechanism for flow of water into and out of the rumen to maintain osmotic balance. An increase in the fractional absorption rates of VFA due to a decrease in pH and an increase in the rumen volume would decrease the osmotic pressure in the rumen.

Several models have been proposed to explain the mechanisms of absorption of VFA (Ash and Dobson, 1963; Argenizo, 1988; Gabel et al., 1989; Dijkstra, 1993). Except for the model proposed by Stevens, (1970) all models assume that transport of sodium across the rumen epithelium supplies the protons for conversion of VFA anions to their acidic form. Ionized form of VFA (VFA^-) does not diffuse passively across the rumen cell membrane (Bugaut, 1987; Gabel et al., 1989). Dijkstra et al., (1993) showed that at neutral pH most (approx. 99 %) of the VFAs are in a dissociated state, yet a considerable fractional absorption of VFA takes place due to supply of protons. Several studies concluded that a decrease in pH increases fractional absorption rate of VFA (Aafjes, 1967; Weigand et al., 1972; Macleod and Ørskov, 1984; Dijkstra et al., 1993). Dijkstra et al., (1993) showed that increases in the fractional absorption were significantly affected by the type of VFA and pH and indicated that at alkaline pH, relative rates of absorption of these three VFA did not differ to such an extent as in case of acidic pH (i.e. levels of pH).

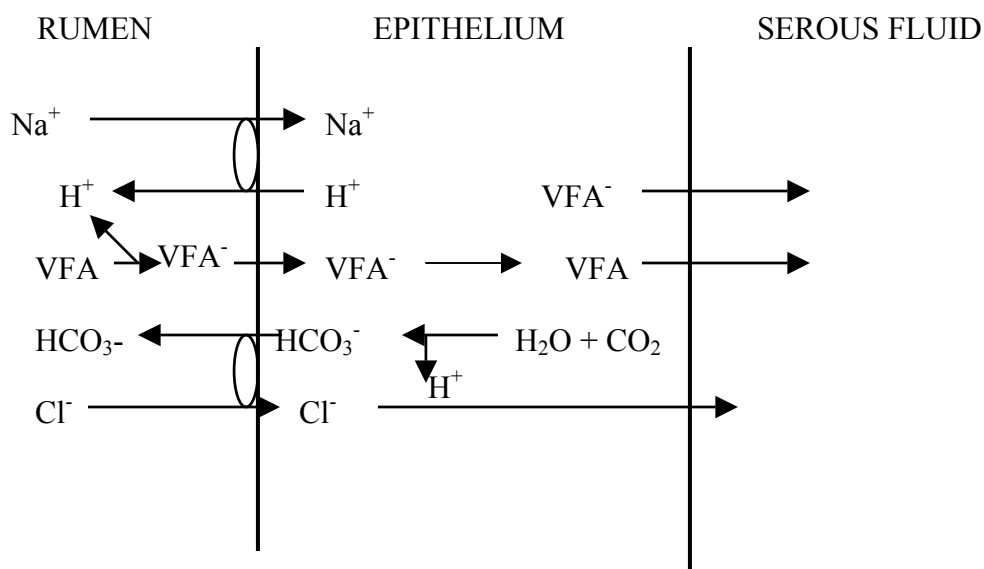


Figure 1.2 Model of transport of VFA and ions across reticulo-rumen epithelium (adapted from Gabel and Martens, 1989)

Acetate absorption from the rumen is dependent upon its concentration in the rumen fluid unlike the case with butyric acid (Dijkstra, 1993). Moreover, with increase in volume of rumen there is a decrease in the effective surface area, which decreases VFA absorption rate. Contribution of VFA passage rate to rate of VFA disappearance is 30 % in cattle at maintenance and 40 % at four times the maintenance (Dijkstra, 1993).

Despite significant advances in our knowledge about rumen metabolism little is known about the role of Na^+ in rumen metabolism especially VFA production and absorption rate (Dijkstra 1993), LDR (Stokes, 1986), and the differential effect of buffers on varying concentration of forage in diet.

Role of Microbes in Mineral Uptake in Rumen

Minerals play an important role in microbial metabolism in the rumen (Durand and Kawashima, 1980). Microbes require minerals for maintaining their osmotic pressure, buffering capacity, protein synthesis and above all, transport of essential nutrients. Though several studies have estimated the requirements of major minerals for microbes, to our knowledge, none has tried to evaluate the effect of mineral uptake by microbes on rumen fluid SID. Moreover, as microbes can be divided into two major fractions according to their properties of attachment to feed (liquid associated bacteria, **LAB**; and solid associated bacteria, **SAB**), these two fractions should flow at different rates. So, in this portion, the differences between microbial mineral composition, their attachment to feed particles, nutrient transport mechanisms and basic function and requirement of mineral in microbes have been reviewed.

Microbial Attachment to Feed

Rumen microbes rapidly attach to the recently ingested feed particles (Cheng et al., 1983, Craig et al., 1987). Attachment to the substrate is accomplished through specific mechanisms requiring adhesions, receptors and non-specific mechanisms that rely on physico-chemical forces such as van-der Waals forces (Pell and Schofield, 1993). Bacteria and protozoa often attach to plant tissues within 5 min of ingestion (Bonhomme, 1990). Rumen microbes can be classified based on their functional interaction with feed particles as: (1) those associated with rumen fluid, (2) those loosely attached to feed particles, and (3) those firmly attached to feed particles (Czerkawski and Cheng, 1988). Microbial population in rumen fluid has little to do

with the digestion of the insoluble fraction of the feed (Lantham, 1980), but is important for initiation of digestion of newly ingested feed particles (McAllister et al., 1994). Microbes attached to feed particles constitute 70 to 80 % of the microbial matter in the rumen (Craig et al., 1987) and are responsible for 80 % of endoglucanase activity, 70 % of amylase activity (Minato et al., 1966), and 75 % of protease activity (Brock et al., 1982) in the rumen. Moreover, hemi-cellulase and cellulase activities are notably higher in the particulate fraction of rumen contents than in fluid associated fraction. Micro-organisms closely associated with feed particles obtain a large proportion of nutrients released from the digestion of feed particles, while the organisms in fluid phase must continuously seek out new sources of soluble substrates to fulfill their nutrient requirements (McAllister et al., 1994).

Depending on size, density, and susceptibility to digestion, small feed particles (≤ 2 mm) are generally retained two to three times longer than the fluid (Owens and Goetsch, 1986). Microorganisms attached to feed particles prolong the residence time of particles in rumen; this is reported to increase the amount of energy partitioned by microorganisms toward maintenance and decrease the efficiency of microbial protein synthesis (Russell and Wallace, 1988). Prolonged residence time is necessary for slowly growing microbes such as protozoa (generation time 5 to 14 h, Williams and Coleman, 1988) and fungi (generation time 24 to 30 h; Joblin, 1981).

Microbial Feed Digestion In-Side Out Concept

Attachment of microbes to nutrient sub-strata is the initial step in the digestion of feed particles in the rumen. A cuticle layer almost resistant to attachment protects forages and cereal grains from microbial degradation (McAllister, 1990). Microbes

gain entry to the readily digestible inner tissue through stomata, lenticels or damaged areas and digestion proceeds from the inside out (Cheng et al., 1991). Therefore, mechanical disruption of forage is the major means for allowing microbial access to the nutrient rich inner tissues. Thus, feed digestion by rumen organisms can be described essentially as an inside-out process where, the nutritionally poor outer plant tissue are attacked to initiate much more slowly than the more easily digestible inner tissues.

Nutrient Transport

The reticulo-rumen can be regarded as a discontinuous fermenter in which the carbohydrates, proteins and lipids of feed are degraded to produce VFA, methane, ammonia, CO₂ and microbial mass. Rumen fluid consists of an enormous amount of bacteria and protozoa, with bacterial counts reaching up to 10¹⁰ per ml and protozoal counts reaching more than 10⁶ per ml (Hungate, 1966). Rumen bacterial growth is dependent on availability of a suitable carbon and energy source. Extracellular enzymes must degrade complex polymers in feeds to low molecular weight compounds to fulfill the nutrient growth requirements of bacteria. The types of microbial population that develop at all of the various locations in the ruminant digestive tract are complex, and depend on the chemical micro-environments provided by nutrients or tissue substrata (Cheng and Costerton, 1980). For bacterial growth to occur, soluble substrates must be transported across the membrane barrier that separates the extra-cellular medium from the cell interior.

Five carrier mediated soluble carbohydrate transport mechanism have been identified in bacteria: (1) facilitated diffusion, (2) shock sensitive system, (3) proton

symport, (4) Na^+ co-transport, and (5) phosphoenolpyruvate-dependent phosphotransferase system. Amino acid transport in rumen bacteria occurs through (1) passive diffusion/facilitated diffusion (Russell and Strobel, 1987), (2) Na^+ dependent mechanisms (Russell et al., 1988), and (3) hydrolysis of chemical bond (ATP).

Influence of Minerals on Rumen Microbial Population

Minerals contribute to the regulation of the physico-chemical characteristics of rumen medium such as osmotic pressure, buffering capacity, redox potential and dilution rate. Durand and Kawashima, (1980) stated that in vitro cellulolysis was not significantly inhibited until the osmolality of culture medium was raised above 400 *mOsmol/kg*; beyond this value differences in the extent of inhibition were thought to be related to the compounds used in the culture medium. Reduction in digestibility of nutrients was found to be greater with poly-ethylene glycol than with NaHCO_3 and KHCO_3 , which can be attributed to the bacterial requirement of Na^+ and K^+ (Carter and Grovum, 1990) for growth and metabolism.

Most predominant rumen bacteria seem to contain enzymes whose activity requires K^+ (Caldwell et al., 1973). Potassium ion content for *Bacillus spp.* is 40 to 49 g/kg DM for *Bacillus* and for *Aerobacter aerogenes* is 16 g/kg (Durand and Kawashima, 1980). Sodium ion is present in substantial amounts in resting bacteria, but almost absent from bacteria except halophiles actively growing in media of moderate salinity (Caldwell et al., 1973). Martinez (1972) found 21 g K^+ and 36 g Na^+ per kg DM in rumen bacterial sediment. High Na^+ content might be related to the fact that some rumen bacteria have been shown to be slightly halophilic (Caldwell et al., 1973). It is assumed that about 94 % of the rumen Na^+ and 20 % of the rumen

K comes from saliva (Martinez, 1972). Previous results have shown that higher concentrations (0.5 to 1.5 g/L) of available K^+ and Na^+ are required for optimum fermentation in the rumen; but it has not been established how much is required by micro-organisms per se and how much of these minerals are required to optimize external physico-chemical factors of rumen micro-organisms.

Phosphorus is necessary for carbohydrate fermentation in the rumen and is a constituent of primary cell metabolites like as nucleotides and coenzymes such as flavin phosphates, pyridoxal phosphates and thiamine pyrophosphate. Phosphorus content of nucleic acid is 10.03 % in DNA and 9.64 % in RNA. Changes in growth rate of microbes affects the P content of the cell (Tempest, 1972). About 80 % of total P in rumen bacteria is contained in the nucleic acids and about 10 % in phospholipids (Van Nevel and Demeyer, 1977). Total P content of the rumen microorganisms may range from 2 to 6 % on a dry weight basis (Kaufmann, 1976). A mean level of about 100 mg/L of available P in the rumen is adequate for growth of rumen bacteria and for cellulolytic activity during in-vitro experiment (Durand & Kawashima, 1980). An in-vitro study for Ca-P-Mg interactions on cellulose digestion suggested that P requirement of rumen bacteria increased as the calcium (Ca) and magnesium (Mg) concentration in the medium rose (Yano & Kawashima, 1979). Moreover, Komisarczuk et al. (1987) reported an overall decrease in protein synthesis by 45 % in cases of P deficiency. Phosphorus deficient diets cause drastic reduction in ATP concentration in the rumen (Komisarczuk et al., 1987) and this effect is probably due to the reduction in microbial catabolic activity (Komisarczuk et al., 1986) and in size of the microbial population in the rumen.

Magnesium is one of the major intra-cellular cations after K^+ in bacteria (about 6 g/kg DM in bacilli) while Ca^{2+} is considered to be low (ranging from 0.1 to 0.3 g/kg DM for bacilli) (Durand and Kawashima, 1980). Magnesium is required for many intra-cellular processes in the rumen micro-organisms (Weinberg, 1977), most of which are associated with ribosomes, while microbial Ca^{2+} functions at the cellular membrane or external to the membrane of rumen microbes (Martinez, 1972). The transport of Mg^{2+} and Ca^{2+} into the cell is an active and energy-dependent process (Durand and Kawashima, 1980). Ca^{2+} and Mg^{2+} are implicated in the control of cell growth and division of the microbes (Hughes and Poole, 1989). But Tempest (1968) measured the Ca content of bacteria and reported that Ca levels did not correlate with the changes in RNA, Mg^{2+} , K^+ , and P but with total cell wall content of the microbes.

The main function of S in relation to rumen microorganisms is to support the synthesis of S-containing amino acids, methionine and cystine. Sulfur is also involved in synthesis of vitamins (thiamine, biotin) and coenzymes. Microbes utilize inorganic sulfate by reducing sulfate to sulfide, which can be incorporated into the microbial S-containing amino acids. Sulfide is rapidly absorbed through the rumen wall and is a function of sulfide concentration in the rumen. Kennedy and Milligan (1978) showed that up to 40 % of the dietary S with an alfalfa based diet, and most of the supplementary methionine S can be taken up by microbes. Microbial biomass can contain up to 8 g of sulfur /kg of DM, of which a large proportion is found in microbial protein (Bird, 1973). Sulfur of the microbial protein can be derived from rumen sulfide pool when animals are fed protein-free diets (McMeniman, 1975) but a significant portion comes from preformed S containing amino acids in the diet

(Harrison and McAllan, 1980). Harrison and McAllan (1980) reported mean protein N: protein S ratio of the mixed rumen bacteria to be 18.5:1.

Chloride transport into the microbial cell might be regulated by a KCl co-transporter mechanism to maintain a trans-membrane electrochemical gradient and possibly to regulate the intra-cellular pH of the microbial cells.

Effect of Rumen pH on Microbial Population

Low ruminal pH reduces fiber digestion in the rumen (Hoover, 1986) by reducing the population of fibrolytic fermenters. Reduction in fiber digestion has been attributed to either reducing the ability of fibrolytic bacteria to attach to feed particles (Cheng et al., 1977) or to slow replication rate of fibrolytic bacteria at low rumen pH (Russell and Dombrowski, 1980). Dombrowski (1980) indicated that one of the main reasons for the elimination of rumen fibrolytic bacteria at low pH environment was an increase in the cost of maintenance of bacteria due to the energy requirement to maintain a proton motive force across the membrane, which eventually results in wash out of fibrolytic population from the rumen. Also, a reduction in crude protein degradation at low pH has been reported (Hoover et al., 1984). Reduced protein degradation is related to reduction in the digestibility of fiber associated with the protein within feeds in the rumen. Undigested fiber associated with feed will reduce access of bacteria and enzymes to the protein and therefore reduce protein degradation in the rumen (Wallace and Cotta, 1989). Efficiency of microbial protein synthesis is affected only when the rumen pH decreases below 5.5 (Hoover and Miller, 1992).

Chapter 2: QUANTIFYING EFFECT OF DIETARY SODIUM BICARBONATE ON RUMEN pH

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Abstract

Dairy cattle are fed sodium bicarbonate (NaHCO_3) to reduce fluctuation in rumen pH. However, the actual mechanism by which NaHCO_3 regulates rumen pH is unclear. A meta-analysis was conducted using studies where NaHCO_3 was added to the diet. Data compiled from 16 available studies with Holstein cows or steers were used to evaluate the effect of dietary NaHCO_3 on rumen pH, volatile fatty acids and calculated strong ion difference (SID) of rumen fluid. Data were analyzed for effect of added levels of NaHCO_3 and the random study effect. Addition of NaHCO_3 to the diet increased rumen pH ($P < 0.001$) and rumen SID ($P < 0.01$). There was no effect of adding NaHCO_3 on rumen volatile fatty acids. A compartmental model was proposed to predict changes in rumen Na^+ on addition of NaHCO_3 . There were two zero-order inputs to the rumen Na^+ pool (i.e. salivary Na^+ flow and dietary Na^+ intake). Rumen Na^+ outflow was considered first order with respect to the quantity of rumen Na^+ . The model predicted changes in rumen SID as affected by changes in dietary NaHCO_3 , and the changes in SID were used to predict changes in rumen pH. Rumen fractional outflow rate was 0.16 /h (SE = 0.06) for Na^+ equivalent. Flow of salivary Na^+ was not affected by addition of NaHCO_3 in the diet. There was no mean bias or linear bias for SID (RMSPE = 0.27) or pH (RMSPE = 0.15). Predictions from formulation data set demonstrated that the model accurately estimated rumen SID

concentration and pH in the data set. The model can be used to predict effect of dietary NaHCO_3 on rumen pH for a given starting pH.

Introduction

Low rumen pH depresses feed intake and fiber digestion and may lead to metabolic disorders (Rogers et al., 1982). However, factors affecting rumen pH and the mechanism of rumen pH control are poorly understood. Addition of sodium bicarbonate (NaHCO_3) or potassium bicarbonate (KHCO_3) counters the decrease in rumen pH observed with high-concentrate diets (Erdman, 1982). Several different mechanisms can explain this effect, (1) sodium (Na^+) or potassium (K^+) ions may increase the strong ion difference (**SID**) of rumen fluid while added bicarbonate is eventually eructated out from the rumen (Kohn & Dunlap, 1998), (2) volatile fatty acid (**VFA**) absorption may be enhanced by sodium-dependent transport resulting in lower VFA concentration in the rumen (Gabel and Martens, 1989), or (3) sodium or potassium salts may increase the liquid dilution rate (**LDR**) thus, decreasing time available for starch digestion in the rumen, and therefore decreasing VFA concentration in the rumen (Russell and Chow, 1992). All of these mechanisms seem plausible given the current analyses. However, there has been no analysis completed to date that can differentiate which explanations are valid.

Kohn (2000) adapted the SID theory (Stewart, 1983) to the rumen milieu, and stated that rumen pH is dependent on rumen VFA concentration, partial pressure of carbon dioxide (pCO_2) and SID (difference between the sum of soluble cations and soluble anions) concentration in the rumen. Therefore, the objectives of this research were: 1) to explain quantitatively the effect of addition of NaHCO_3 on rumen pH,

VFA concentration and SID, and 2) to propose a mathematical model that can predict the effect of adding NaHCO₃ on rumen pH.

Material and Methods

Compilation of Literature

Treatment means from 16 studies on effect of NaHCO₃ on rumen pH with Holstein cows and steers were used (Table 2.1). Articles published from 1962 to 1999 were selected when the amount of NaHCO₃ consumed, rumen pH, and VFA concentrations were reported for rumen- cannulated cows. Studies where rumen fluid was collected via rumino-centesis or a stomach tube were not included. There were 40 treatment means from 16 studies, with treatments being control and varying quantity of NaHCO₃ in the diet. Most of these studies were aimed at investigating the effect of adding dietary buffers on rumen pH.

Equations used in Meta-analyses

Using the published values of rumen pH and VFA concentration (M), various parameters were estimated using the following equations:

$$[H^+] = 10^{-pH} \quad (1)$$

where $[H^+]$ represents hydrogen ion concentration (M); and

$$[OH^-] = k_w / [H^+] \quad (2)$$

where k_w represents the dissociation constant of water ($\text{mol}^2 / \text{L}^2$), and $[OH^-]$ represents hydroxyl ion concentration (M). Rumen $[HCO_3^-]$ (M) was calculated using Henderson-Hasselbalch equation:

$$pH = pK_a + \text{Log} [HCO_3^-] / pCO_2 \quad (3)$$

where $p\text{CO}_2$ represents partial pressure of carbon dioxide, which was assumed to be 0.65 atm for high concentrate diets and 0.55 atm for high forage diets (Barry et al. 1977), and pK_a was assumed to be 7.74 (Kohn and Dunlap, 1998). Equation (3) can be rearranged to:

$$[\text{HCO}_3^-] = (10^{(\text{pH}-\text{p}K_a)}) \times p\text{CO}_2 \quad (4)$$

Rumen VFA anion (VFA^-) concentration (M) was calculated using the Henderson-Hasselbalch equation as follows:

$$\text{pH} = \text{p}K_a + \text{Log} [\text{VFA}^-]/[\text{VFA}^0] \quad (5)$$

where $[\text{VFA}^-]$ and $[\text{VFA}^0]$ are the concentrations (M) of dissociated and undissociated VFA; pK_a for VFA in rumen is 4.8. Equation (5) can be rearranged to:

$$[\text{VFA}^-] = 10^{(\text{pH}-\text{p}K_a)} \times [\text{VFA}] / ((10^{(\text{pH}-\text{p}K_a)}) + 1) \quad (6)$$

Using the above calculated parameters rumen SID concentration (M) was calculated using the equation:

$$[\text{SID}] = [\text{VFA}^-] + [\text{HCO}_3^-] + [\text{OH}^-] - [\text{H}^+] \quad (7)$$

where $[\text{SID}]$ represents SID concentration in the rumen (M), and other terms are as defined previously. Rumen SID (mol) was calculated by multiplying rumen SID concentration with the rumen volume (L).

Statistical Analysis on Pooled Data

Data were analyzed using the Fit Model procedure of JMP (SAS Inst., Cary NC). A mixed model analysis was performed on Y variables: rumen pH, rumen SID and rumen VFA concentrations as follows:

$$Y_{ij} = \mu + T_i + S_j + T_i \times S_j + e_{ij}$$

where μ is the overall population mean, T_i is the effect of addition of NaHCO_3 to the diet (mol d^{-1}), S_j is random study effect, $T_i \times S_j$ is the interaction between NaHCO_3 and study and e_{ij} is the residual error. No effect of study \times treatment interaction was found so it was not included in the final model. Correlation coefficients reported did not include the correlation due to the random study effect.

Model Formulation

Fourteen studies were used to develop a compartmental model for prediction of rumen pH. Two studies were excluded (Emery et al., 1961; Rogers et al., 1979) from the model as the amount of NaHCO_3 used in these studies was more than four times what is commonly fed today. A single compartment model was used to estimate the fractional rate of removal of Na^+ equivalent (d^{-1}) and the change in salivary Na^+ flow (mol d^{-1}) due to addition of NaHCO_3 in the diet. The model is shown in Figure 2.1. Two inputs were assumed to contribute to the quantity (mol) of Na^+ in the rumen (Na_r^+): dietary Na^+ (mol d^{-1}) and salivary Na^+ (mol d^{-1}). Sodium ion disappearance (mol d^{-1}) by passage and absorption would equal the fractional disappearance rate (d^{-1}) times the quantity (mol) of Na^+ in the rumen. Thus, the following differential equation describes the change in rumen sodium (mol d^{-1}) per unit time:

$$d\text{Na}_r^+/dt = \text{Dietary Na}^+ + \text{Salivary Na}^+ - k_d \times \text{Na}_r^+ \quad (8)$$

where, Na_r^+ represents the quantity of Na^+ in the rumen (mol); dietary Na^+ (mol d^{-1}) is the amount of Na^+ in diet fed per day; salivary Na^+ (mol d^{-1}) is the amount of Na^+ coming from saliva per day; and k_d (d^{-1}) represents the fractional rate of disappearance of Na^+ from the rumen.

The studies used for this analysis reported average rumen concentrations after animals had adjusted to diets. Assuming the change in Na^+_r has reached a steady state:

$$d\text{Na}^+_r/dt = 0 \quad (9)$$

the two inflows into the rumen Na pool will equal the outflow from the rumen

$$\text{Dietary Na}^+ + \text{Salivary Na}^+ = k_d \times \text{Na}^+_r \quad (10)$$

Rearranging equation (10)

$$(\text{Dietary Na}^+ + \text{Salivary Na}^+) / k_d = \text{Na}^+_r \quad (11)$$

This equation resembles that of a straight line: where the intercept is salivary Na^+ / k_d and the slope is $1/k_d$. This line was fit for data from Staples (1989) as the quantity of dietary Na^+ (mmol d^{-1}) and rumen Na^+ (mM) concentration were reported for all treatments.

Actual dietary Na^+ was not reported in most of the studies used in this analysis; however, each study included a control diet without NaHCO_3 , and similar diets with varying amounts of NaHCO_3 . Therefore, we calculated change in dietary Na^+ across treatments within each study. Rumen Na^+ concentrations were not usually reported. Therefore, the change in rumen SID (ΔSID) was calculated across treatments (equation 7), and this change was assumed to result from changes in Na^+ in the rumen. Because ΔSID may have also resulted from effects of Na^+ on concentrations of other ions like Cl^- or VFA^- , it is called Na^+ equivalent. The quantity of Na^+ equivalent relative to the control treatment was calculated for each study as the ΔSID ($\text{mol equivalents L}^{-1}$) times the rumen volume (L). Thus, the following regression equation enabled us to estimate changes in saliva Na^+ flow (mol

d⁻¹) due to addition of NaHCO₃ and fractional disappearance rate (k_d) of Na⁺ equivalent:

$$(\Delta\text{Diet Na}^+ + \Delta\text{Saliva Na}^+) / k_d = \Delta\text{SID} \times V \quad (12)$$

where $\Delta\text{Diet Na}^+$ represents the change in intake of Na⁺ (mol d⁻¹) relative to the control (no NaHCO₃ diet); ΔSID represents the change in flow of SID (mol equiv L⁻¹ d⁻¹) calculated from reported values of pH and VFA data (equation 7), V represents rumen volume (L), k_d is fractional disappearance rate, and $\Delta\text{Saliva Na}^+$ is the change in Na⁺ flowing with saliva (mol d⁻¹) due to the addition of NaHCO₃. When rumen volume was not reported, we assumed it was equal to the average of the rumen volumes (46 L) for the studies that reported it. The $\Delta\text{SID} \times V$ was regressed on $\Delta\text{Diet Na}^+$. Study was also included in the model as a random effect, and the slope and intercept were fit using a mixed model (St-Pierre, 2001). The k_d and ΔSaliva were calculated from the slope and intercept.

Model Predictions

Predicted SID was calculated using the model equation (12) and values were fit for k_d and $\Delta\text{Saliva Na}^+$. The predicted rumen pH was calculated using Henderson-Hasselbalch equation assuming that equi-molar proportions of HCO₃⁻ and Na⁺ had been added to the rumen. Model predictions were then compared with residual (predicted – observed) values from the dataset. Mixed model analysis with random study effect was performed for residuals against predicted values and other selected variables. Mean and linear biases were determined from model predictions (Bibby and Toutenburg, 1977). Root mean square prediction error (**RMSPE**) was calculated from the following equation (Bibby and Toutenburg, 1977):

$$\text{RMSPE} = \sqrt{\{(\text{predicted} - \text{observed})^2 / n\}}$$

The fraction of variance attributed to mean bias was calculated as the mean bias squared divided by the mean square prediction error (**MSPE**), and the fraction attributed to remaining dispersion was residual error²/MSPE. Mean square prediction error attributed to the study effect was estimated as the partial sum of squares for the study divided by the sum of all partial sums of squares and the error sum of squares.

Model prediction of k_d was compared with published values for rate of passage (k_p). We computed mean and standard error for k_d (0.16, S.E = 0.06) and k_p (0.089, S.E = 0.02) from the slope of model equation and from 13 published values of rate of passage, respectively. Student's t distribution was used to compare k_d to k_p and was considered to be different when

$$\text{Mean of } (k_d - k_p) > t_{df,\alpha} \times \delta_{\text{diff}} \quad (\text{Steel et al., 1997})$$

where k_d is model predicted rate of disappearance; k_p is mean calculated rate of passage from the published values; Student's t-distribution with degree of freedom calculated for independent samples and unequal variance between k_d and k_p , by weighing them for unequal variance was as follows:

$$(s_d^2/n_d + s_p^2/n_p)^2 / [(s_d^2/n_d)^2/(n_d-1)] + [(s_p^2/n_p)^2/(n_p-1)] \sim 19 \quad (\text{Steel et al., 1997})$$

where s_d^2 is variance due to fractional disappearance rate of Na^+ ; n_d and n_p are the number of data points used to calculate disappearance and passage rates respectively; s_p^2 is the variance due to fractional passage rate of Na^+ from the rumen; $t_{df, 0.05}$ for 19 degree of freedom is 1.73.

δ_{diff} represents standard error of difference between disappearance and passage rate of Na^+ calculated as

$$(k_d - k_p) = \sqrt{((\delta_{ed})^2 + (\delta_{ep})^2/r_p)}$$

where δ_{ed} represents standard error associated with model prediction; δ_{ep} represents standard error associated with mean calculated from published literature values; r_p represents the number of data points. If mean $(k_d - k_p)$ was greater than 1.73, it was assumed that k_d was greater than k_p or else there was insufficient evidence to state the difference between k_d and k_p .

Statistical analyses were performed with JMP 5.0 (SAS Inst., Cary NC), and statistical significance was declared at $P < 0.05$ unless otherwise stated.

Flow of Strong Ions

Staples et al. (1989) reported the mineral concentration in rumen fluid for various treatments (basal diet, 1 % NaHCO_3 , 1 % multi-element buffer, and 3 % multi-element buffer). The means from this data were used to calculate the amount of mineral flowing from saliva into the rumen per day and fractional rate of removal of minerals from the rumen per day. Dietary Na^+ (mmol d^{-1}) was calculated from reported values (g/d). Rumen Na^+ (mmol) was regressed against dietary Na^+ . Fitting the treatment means from the dataset (Staples et al. 1989) to equation (11), the fractional removal rate of Na^+ from the rumen was calculated as:

$$\text{Na}_r^+ = (\text{Dietary Na}^+ + \text{Salivary Na}^+) / k_d$$

where Na_r^+ is the quantity of Na^+ in rumen fluid (mmol) and is equal to the concentration of Na^+ in the rumen multiplied by rumen volume; Dietary Na^+ represents intake of Na^+ from diet (mmol d^{-1}); k_d is the fractional rate of disappearance of Na^+ from rumen (d^{-1}); Salivary Na^+ represents amount of Na^+ coming from saliva (mmol d^{-1}).

Published values for concentration of Na⁺ (mM) in saliva reported by McDougall, (1948) were used in the following equation. Volume of saliva (L) was calculated as:

$$\text{Volume of Saliva} = (\text{Salivary Na}^+ / k_d) / \text{Na}_s^+$$

where Salivary Na⁺/k_d is the quantity of saliva coming into rumen (mmol) and is equivalent to the intercept of the above regression equation (11); Na⁺_s represents reported value of Salivary Na⁺ concentration (mM) by McDougall, (1948).

Salivary mineral ion (mmol d⁻¹) was calculated by salivary mineral concentration (McDougall, 1948) times the calculated salivary volume (L). The fractional rate of disappearance of other minerals from rumen was calculated using the following equation:

$$k_d = (D+S) / \text{Mineral}_r \quad (13)$$

where k_d represents the fractional rate of disappearance of mineral (d⁻¹); D represents amount of mineral coming from diet per day (mmol d⁻¹); S represents the amount of mineral ion coming from saliva per day (mmol d⁻¹); Mineral_r is the quantity of mineral ion in rumen fluid (mmol) and represents the concentration of mineral ion in rumen fluid multiplied by rumen volume.

Results

Meta-analysis

Rumen pH increased with addition of NaHCO₃ ($P < 0.001$; $r^2 = 0.25$) and differences were noted among studies ($P < 0.001$), while no study by NaHCO₃ interactions were detected (Figure 2.2). Addition of dietary NaHCO₃ increased the rumen SID concentration ($P < 0.001$; $r^2 = 0.17$), and there was a study effect ($P < 0.001$), but no study by NaHCO₃ interaction (Figure 2.3). The addition of dietary

NaHCO₃ did not effect the rumen VFA concentration (Figure 2.4). There was a study effect on VFA concentration ($P < 0.001$) but no interaction of study with NaHCO₃.

Model Predictions

We found a linear increase in SID when the Δ SID was fit against Δ Na⁺ in the diet due to addition of NaHCO₃ (Figure 2.5). The equation of the regression line was as follows:

$$\Delta\text{SID} \times V = 0.26 \text{ (SE} = 0.1) \times \Delta\text{Dietary Na}^+ - 0.21 \text{ (SE} = 0.14)$$

where $\Delta\text{SID} \times V$ is Δ SID times the rumen volume (mol), which is equal to Δ Na⁺ in the rumen (mol); $\Delta\text{Dietary Na}^+$ is the change in amount of Na⁺ coming from diet per day (mol d⁻¹); and intercept is amount of Na⁺ coming from saliva per day (mol d⁻¹) divided by fractional disappearance (d⁻¹) of Na⁺ from the rumen. The intercept for the regression equation is a function of change in Na⁺ coming from saliva on addition of NaHCO₃, but it was not significantly different. Hence, we conclude that addition of dietary buffer did not affect the flow of saliva to the rumen. The coefficient of regression is an inverse function of the fractional out-flow rate (k_d) from the rumen which equaled 0.16 /h (SE = 0.06). Previous research with dairy cattle (Staples et al., 1989) has shown rumen passage rate (k_p) of 0.093 /h. The difference between the rate of disappearance and passage of Na⁺ suggests that some of the change in SID as predicted by the dietary change was due to absorption or secretion of ions. Some of the additional Na⁺ may have been absorbed or the addition of NaHCO₃ may have affected absorption of Cl⁻.

Model Evaluation

Figure 2.6 shows the model predictions compared with residual (predicted – observed) values for Δ SID. For SID (mol) RMSPE was 0.27 (mol). There was no significant mean or linear bias. Mixed model analysis revealed that most of MSPE was due to variation among experiments ($P < 0.01$).

In the model we assumed that Δ SID is equal to ΔNa^+_r . So, this ΔNa^+_r is equal to change occurring due to addition of NaHCO_3 to the diet. The predicted rumen pH was calculated using Henderson-Hasselbalch equation assuming that equi-molar proportions of HCO_3^- and Na^+ were added to the rumen, and HCO_3^- diffused out by forming water and CO_2 . The rumen pH was calculated after addition of known amounts of NaHCO_3 in case of high concentrate diets as follows (Kohn and Dunlap, 1998):

$$\text{pH} = 7.74 + \text{Log} (\Delta\text{HCO}_3^-_r / 0.65)$$

where 7.74 is effective pka of bicarbonate; $\Delta\text{HCO}_3^-_r$ is the change in HCO_3^- in the rumen with the assumption of equi-molar change in Na^+_r ; 0.65 is the partial pressure of CO_2 (atm) for high concentrate diets (Barry et al., 1977).

No significant mean or linear bias was found when residuals (predicted – observed) were regressed against predicted rumen pH. Root mean square prediction error was 0.16 for rumen pH (Figure 2.7). Also, the mixed model analysis showed that most (57 %) model error was due to variation among studies ($P < 0.001$).

Flow of Strong Ions

Treatment means from Staples et al. (1989) were used to predict disappearance rate of individual minerals from the rumen (Table 2.2). Fractional

disappearance rate of Na^+ from the rumen (d^{-1}) was calculated from the regression equation:

$$\text{Na}_r^+ = 0.1614 (\text{S.E}= 0.2) \times \text{Dietary Na}^+ + 94.27 (\text{S.E}= 14.04)$$

where Na_r^+ is the concentration of Na^+ multiplied by rumen volume, quantity of Na^+ in rumen fluid (mmol); Dietary Na^+ is intake of Na^+ from diet (mmol d^{-1}); k_d represents fractional rate of disappearance of Na^+ from rumen (d^{-1}); Salivary Na^+ is the amount of Na^+ coming from saliva (mmol d^{-1}). Fractional disappearance rate of Na^+ as calculated from the reciprocal of slope was $0.26/\text{h}$ (Table 2.2).

Calculated volume of saliva flowing into rumen equaled 157 L d^{-1} . Mineral disappearance rate and amount of mineral coming from saliva (mol d^{-1}) flowing into rumen per day are reported in Table 2.2.

Discussion

Meta-analysis identifies general features of a system through the study of pooled data from different published sources. The concept of pooling is utilized to validate the role of NaHCO_3 as source for cations (Na^+) influencing rumen pH.

It is well known that addition of NaHCO_3 increases rumen pH. Several theories have been proposed as a mechanism that can regulate rumen pH, but none have been evaluated quantitatively. Therefore, we conducted a meta-analysis to test various hypotheses regarding mechanisms by which dietary NaHCO_3 affects rumen pH. The increase in dietary NaHCO_3 appears to increase SID in the rumen, which could be due to an increase in cation – anion difference by the presence of Na^+ , and HCO_3^- is removed by eructation (Kohn and Dunlap, 1998). The results of our analysis

indicate that NaHCO_3 does not generally affect rumen VFA that is in agreement with 14 out of 16 studies. However, VFA concentrations were reduced in two studies (Erdman et al., 1982; Staples et al., 1989). A VFA absorption model proposed by Gabel and Martens (1989) suggests a potential Na-H^+ mediated pump which enhances the absorption of the acidic form of rumen VFA. In this analysis, we also found a definite relationship between dietary NaHCO_3 and rumen SID. Thus, we can propose that rumen pH is regulated by increasing SID when additional cations are fed to dairy cattle. The effect of cationic salts may be from directly increasing Na^+ concentration or may also be from altering the rumen outflow rate of various anions (e.g. Cl^-) from the rumen. In this analysis, we found significant study effect for all our dependent variables. Most studies are designed to minimize variation within study by using similar animals or the same animals, but this practice increases the possibility that the sample does not represent a broad population, and therefore samples from different studies are likely to vary more from each other than from within studies.

A simple compartmental model that emphasizes the reasoning behind the effect of dietary addition of Na^+ on rumen milieu is proposed. Two zero order inputs (i.e. dietary and salivary Na^+) and one first order outflow from rumen sodium pool were used to evaluate the changes in Na^+_r . Predictions from the model suggested addition of buffers to the diet does not affect the amount of Na^+ coming from saliva. Fractional outflow rate of Na^+_r from the rumen is 0.16 /h (SE = 0.06), while previously the fractional passage rate has been calculated to be about 0.09 /h (Staples et al., 1989). Analysis of Staples data (1989) suggested that Na^+ and K^+ might affect the disappearance rate of strong anions (Cl^-) from the rumen pool. Thus, the increase

in concentration of strong cations in the rumen might increase the SID by enhancing the removal of anions from the rumen.

Analysis of previously published values of k_p with model predicted k_d suggests that Na^+ exits the rumen by absorption through the rumen wall along with passage lower down the tract. Moreover, relatively higher proportion of Na^+ enters via the saliva, which may be the reason we did not find any changes in salivary Na^+ on addition of buffers to the diet. Mean and linear bias for both SID concentration and pH were not significant. The prediction from the formulation data set demonstrated that the model can estimate rumen SID and pH in this data set.

Implication

The meta-analysis has highlighted the effects of a cationic source (Na^+) on rumen pH. Also, the analysis revealed that NaHCO_3 in the diet generally does not affect the VFA concentration as was proposed earlier. Analysis of model prediction with previous published work suggests Na^+ leaves the rumen through passage and absorption. The model also showed that the addition of NaHCO_3 in the diet does not affect the flow of salivary Na^+ . This model enables us to predict changes in rumen SID and pH for a group of animals when NaHCO_3 is added to a diet.

Table 2.1. Studies used to conduct meta-analysis on the effect of addition of NaHCO₃ on rumen pH fluctuations

Reference	Means	Animals	Amount of NaHCO ₃ ^a	Design ^b
Snyder et al., 1983	4	4	1.20	LS
Kennelly et al., 1999	4	4	1.20	LS
Staples et al., 1988	2	4	1.00	LS
Rogers et al., 1982	2	4	5.00	LS
Erdman et al., 1982	2	4	1.00	LS
Rogers et al., 1979	4	2	0.36 ^c , 0.72 ^c	LS
Stokes et al., 1986a	2	4	0.70	LS
Stokes et al., 1986b	3	3	0.38, 0.68	LS
Emery et al., 1961	2	3	1.00 ^d	LS
Ghorbani et al., 1989	4	6	1.00	RLS
Solorzano et al., 1989	2	3	0.71	LS
Jacques et al., 1986	4	6	1.00	LS
Emmanuale and Staples, 1994	2	4	1.00	LS
Wiedmeier et al., 1987	2	3	2.00	LS
Teh et al., 1985	2	4	1.00	LS

^aNaHCO₃ fed as a % of Dry Matter Intake

^bLS is latin square, RLS is repeated latin square

^ckilogram of sodium bicarbonate fed per day

^dlb of sodium bicarbonate fed per day

Table 2.2. Disappearance rate of minerals and their concentration in saliva (mol d^{-1}) calculated from treatment means from Staples et al., 1989.

Mineral	Disappearance rate (K_d) ^a	Concentration in Saliva ^b
Sodium	0.26	27.99
Potassium	0.23	1.24
Calcium	0.58	0.09
Magnesium	0.32	0.05
Chloride	0.22	1.50
Phosphorus	0.39	2.65

^a fractional disappearance rate of mineral from the rumen (h^{-1})

^b amount of mineral coming from saliva into rumen per day (mol d^{-1})

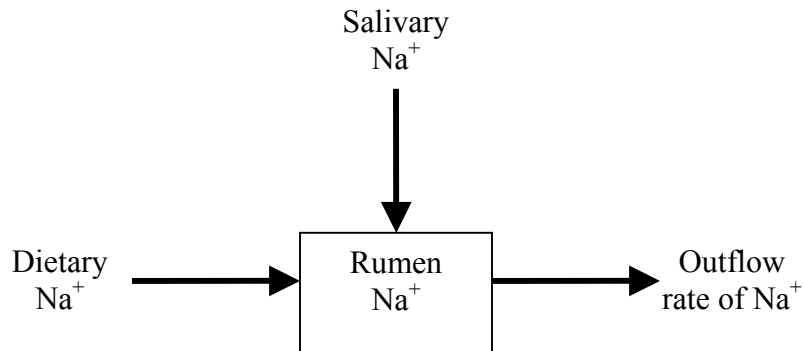


Figure 2.1. Diagrammatic representation of a single compartmental model; where Rumen Na^+ (mol) is the quantity of Na^+ in the rumen; Dietary Na^+ (mol d^{-1}) is the quantity of Na^+ fed per day; Salivary Na^+ (mol d^{-1}) is the quantity of Na^+ coming from saliva per day; outflow rate (mol d^{-1}) is the quantity of Na flowing out of rumen per day.

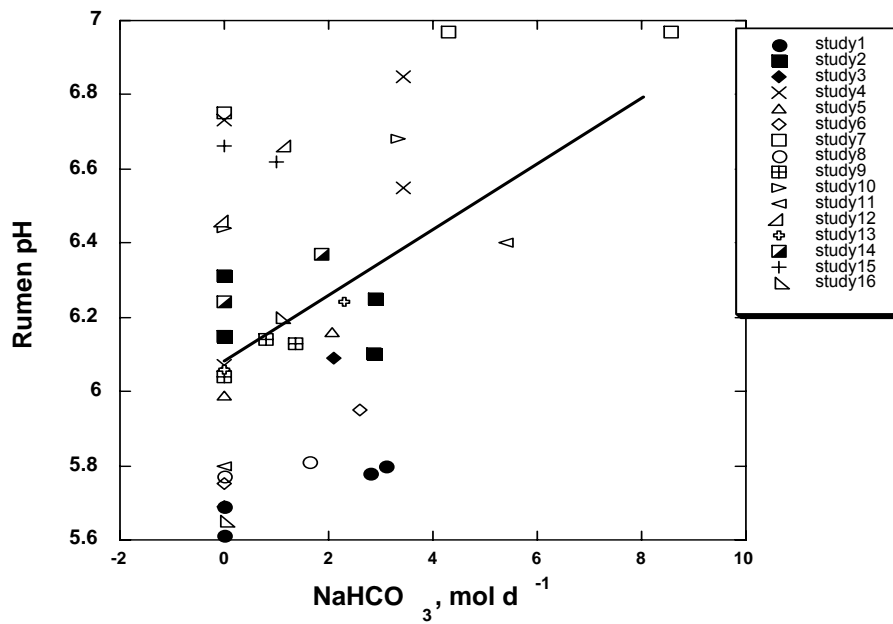


Figure 2.2. Effect of addition of NaHCO_3 on rumen pH. Data used for model development are shown with different symbols representing different studies. Treatment means from published literature were used to evaluate the effect of NaHCO_3 on rumen pH.

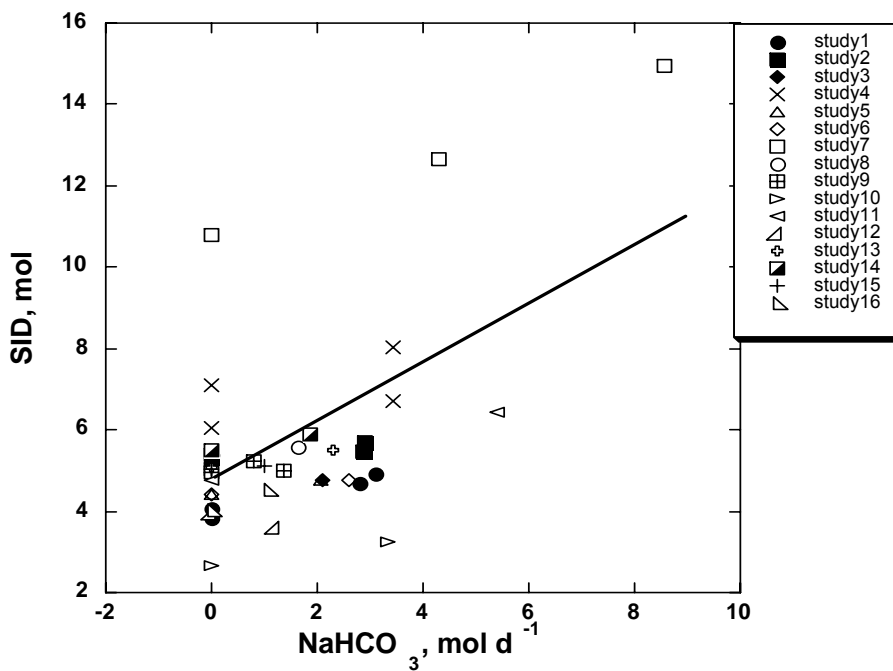


Figure 2.3. Effect of addition of NaHCO_3 on rumen strong ion difference (SID). Data used for model development are shown with different symbols representing different studies. Treatment means from published literature were used to evaluate the effect of NaHCO_3 on rumen SID.

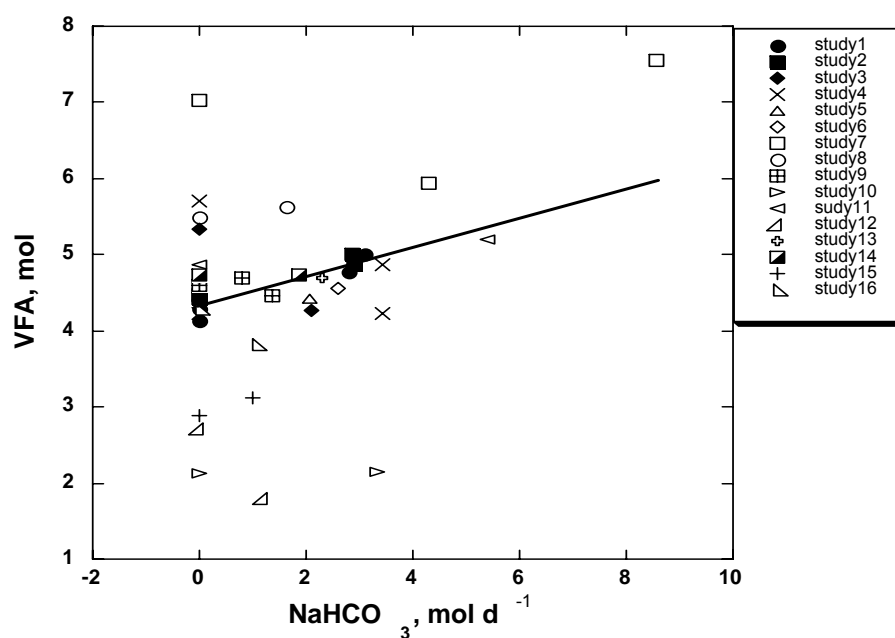


Figure 2.4. Effect of addition of NaHCO_3 on rumen volatile fatty acid (VFA). Data used for model development are shown with different symbols representing different studies. Treatment means from published literature were used to evaluate the effect of NaHCO_3 on rumen VFA.

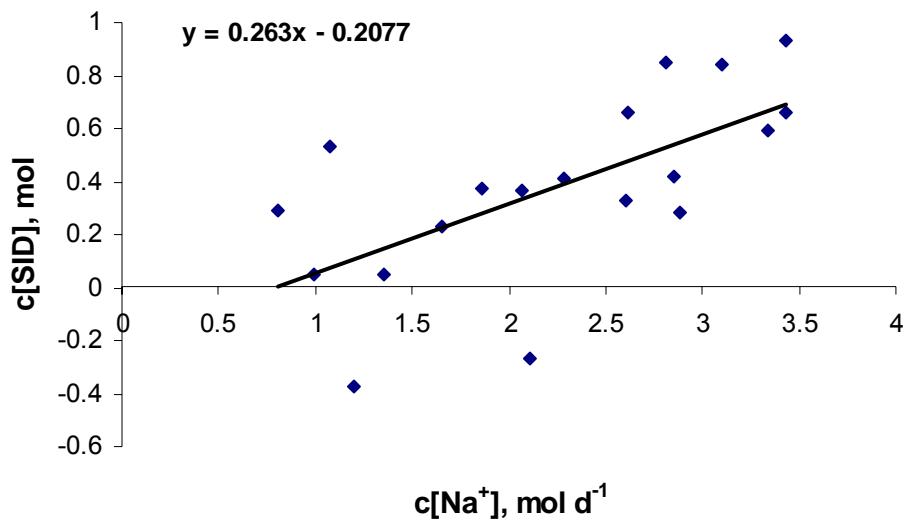


Figure 2.5. Relationship between change in strong ion difference (ΔSID , mol) and change in dietary NaHCO_3 (mol d^{-1}). (\blacklozenge) represents difference between control and treatment means from published literature.

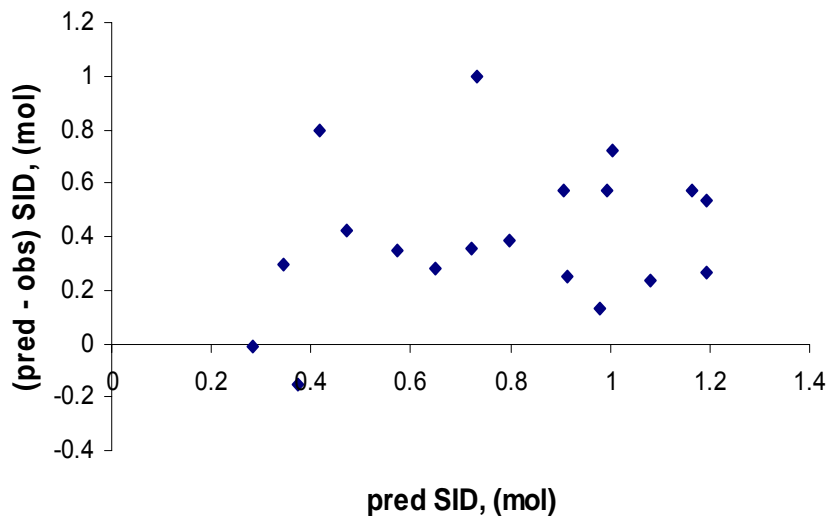


Figure 2.6. Residual (predicted – observed) values compared for the data set for model formulation with predicted strong ion difference (SID , mol); Root mean square prediction error (RMSPE) = 0.27 , mol .

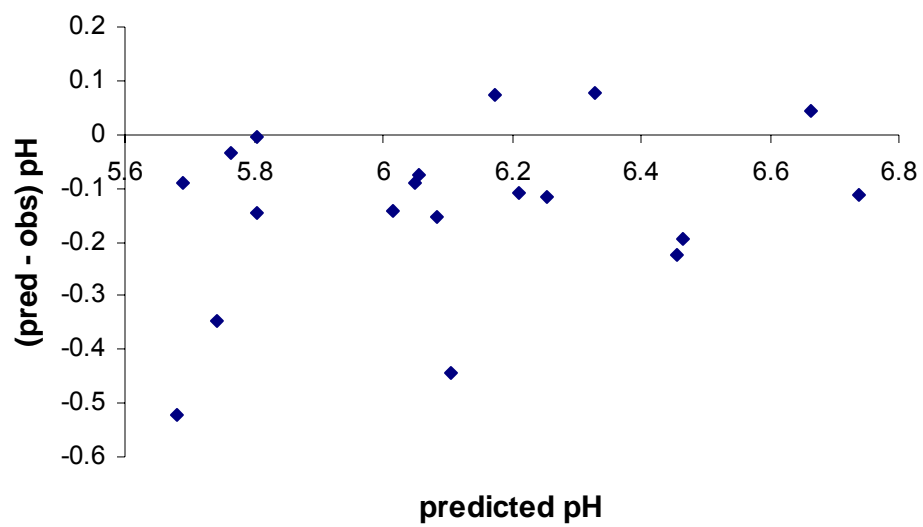


Figure 2.7. Residual (predicted – observed) values compared for the data set for model formulation with predicted rumen pH; Root mean square prediction error (RMSPE) = 0.16.

Chapter 3: EFFECT OF GROWTH CONDITIONS ON MINERAL COMPOSITION OF RUMEN MICROBES

To be submitted to Journal of Animal Science

Abstract

Uptake of cations and anions by rumen microbes may affect rumen strong ion difference (**SID**) and pH. An in vitro study was conducted to determine the macro-mineral composition of solid and liquid associated bacteria in rumen contents. Effects of buffer strength, pH, feed type, and length of incubation were evaluated. Buffer concentration was 0.5x, 1x, 2x normal concentration either at pH 6.8 or adjusted to pH 5.8 at normal concentration of buffer. Buffer with rumen fluid (4:1 vol/vol) was incubated with alfalfa hay or corn grain for 4, 14, or 24 h. Differences were noted at ($P < 0.05$). Ash content was lower for solid than liquid associated bacteria but SID was not different between bacterial pellet due to a corresponding decrease in mineral concentration of Na^+ , Cl^- , S^- , PO_4^{2-} , Mg^{2+} , K^+ and Ca^{2+} in solid associated bacteria. Increase in buffer strength increased ash content but SID was not affected due to a corresponding increase in Na^+ , Cl^- , and PO_4^{2-} . Low buffer pH decreased ash and SID by increasing Cl^- , S^- , PO_4^{2-} and decreasing Na^+ , and Ca^{2+} . In general, microbes took up more cations than anions from the buffer. Ash content was higher for alfalfa hay than corn grain but SID was not affected because the concentration of K^+ , Cl^- , Mg^{2+} , Ca^{2+} and S^- were higher for alfalfa hay than corn grain. Over the length of incubation, ash content decreased, while SID was unchanged as Na^+ , K^+ , and Cl^- all decreased from 4 to 24 h of incubation. The calculated SID of the microbial pool decreased with pH but was not affected by other treatments due to a similar change in uptake of

cations and anions. These results enable prediction of the change in SID due to microbial growth and this change would further affect rumen pH.

Introduction

Minerals may play an important role in regulating strong ion difference (**SID**) in the rumen (Kohn, 2000). Bicarbonate buffers of Na^+ and K^+ , and volatile fatty acids (**VFA**) play an important role in buffering the rumen pH (Kohn and Dunlap, 1998). We conducted a preliminary experiment to quantify the release of minerals from various feeds, but found instead that microbes took up more of some minerals than they released. Other studies have shown that uptake of minerals by microbes depends on the type of feed, length of incubation (Storm & Ørskov, 1983), and ionic concentration of the medium (Poupard et al., 1973).

Availability of nutrients within feedstuffs is dictated largely by the physical structure of the forage or grain being digested (McAllister et al., 1990). Structural differences between types of plant materials affect the microbial processing of forages and grains, and alter the type of microbial population that will develop in the rumen (Cheng et al., 1991). The extent of digestion also depends on forage particle size, as smaller particles not only pass through the rumen faster (Galyean and Owens, 1991) but could also provide more surface area for microbial attachment, and hence, release of nutrients into the rumen liquid. Changes in rumen fermentation are observed after feeding high-concentrate diets due to a combination of substrate and pH effects (Russell, 1998). Presence of higher proportion of non-structural carbohydrates in the diet decreases the rumen pH (Calsamiglia et al., 2001), affecting microbial metabolism and end products of rumen metabolism (Cheng et al., 1991).

The composition of the growth medium influences the morphology of the microorganisms (Poupard et al., 1973) and affects the transport of nutrients (Macleod et al., 1973).

Previous studies have shown that microorganisms associated with the particulate fraction in the rumen constitute a large portion of the rumen microbial population (Merry and McAllan, 1983; Craig et al., 1987). Quantitative measurements of liquid and solid associated microbial populations in the rumen have been reported for nitrogen, nucleic acid, lipid and polysaccharide concentration (Craig et al., 1987 b). However, little has been done to evaluate the mineral concentration of rumen microbes. The objective of the present study was to determine the mineral composition of solid associated bacteria (**SAB**) and liquid associated bacteria (**LAB**) and the effect of osmotic strength of the medium, type of feed, length of incubation, and pH on the mineral composition of microbes.

Material and Methods

Feed and Buffer Preparation

Samples of alfalfa hay and corn grain were dried at 55 °C in a forced-air oven and allowed to air equilibrate before being ground through a 1-mm screen. Six-gram samples of ground alfalfa hay or corn grain were transferred to 500-ml Erlenmeyer flasks (12 for each feed).

The in vitro medium was prepared according to Goering and Van Soest (1970) and adjusted to make four media treatments (2000 mL each) at different osmotic strengths or pH (Table 3.1): one half concentration of all ionic salts (0.5x), normal (1x), or twice normal concentration (2x), and normal concentration but adjusted to pH

5.8 (instead of 6.8) with HCl. Carbon dioxide (CO₂) was perfused through the media in each flask using a bubble disperser until the pH stabilized.

Rumen Fluid Collection

Animal care and use was according to established procedures of the University of Maryland Animal Care and Use Committee. The experiment was conducted from July through August of 2004 and samples were collected from animals housed at University of Maryland campus farm (College Park, MD). Two 5-year old non-lactating Holstein cows fitted with permanent rumen cannulae were used as rumen fluid donors. Cows were fed once daily a mixed diet consisting of grass/legume hay. Cows were fed at 0730 h and rumen fluid (4000 mL) was collected 1 h post-feeding. Rumen fluid was collected in an airtight plastic container and immediately taken to the laboratory.

Sample Preparation

Rumen fluid was filtered through eight layers of cheese cloth and 675 mL of rumen fluid was transferred to a corresponding Erlenmeyer flask containing different concentrations of buffered media. The starting pH of the rumen fluid /buffer mixture under CO₂ was adjusted with 1N HCl or 1N NaOH solution to either 6.8 or 5.8. Rumen fluid/ buffer mixture (350 mL) was transferred to each of the 24 Erlenmeyer flask (500 ml) containing feed samples. Samples (12 for each feed per run) were incubated for 4, 14 or 24 h in a water bath at 39 °C under constant CO₂ pressure. The procedure was repeated on four different days to obtain four replicates per sample, time and treatment combinations.

Isolation of Microbial Population

After incubation, the samples were centrifuged at 500 x g for 5 min. The supernatant obtained was filtered through 11- μ m filter paper (Whatman # 1, Whatman International Inc.) to remove the particulate matter. The filtrate was then centrifuged at 20,000 x g for 20 min and the pellet obtained was re-suspended in distilled water and centrifuged at 20,000 x g for 20 min to obtain the microbial pellet of LAB fraction (Perez et al., 1998).

The filtrant and pellet from the first centrifugation (500 x g) were transferred to Erlenmeyer flasks and about 50 mL of detachment solution (buffered media as used for incubation plus 0.1 % Tween 80 and 0.1 % methyl-cellulose) and 15 glass beads were added to detach microbes from feed particles (Perez et al., 1998). The flasks were maintained at 4 °C for 24 h and were later transferred to a shaking water-bath for 1 h at 39 °C. After shaking in the water-bath for 1 h, the samples were re-centrifuged at 500 x g for 5 min, the pellet obtained was collected and frozen for further analysis while the supernatant fraction was filtered through 11- μ m filter paper and the filtrate was centrifuged twice at 20,000 x g for 20 min to obtain a microbial pellet of the SAB fraction.

Analysis

Dry Matter (**DM**), organic matter (**OM**) and ash (g/100 g) were calculated for SAB and LAB fraction. Feed samples were dried in a forced air oven at 55 °C for 12 h. Microbial pellets were ashed at 550 °C for 12 h in a muffle furnace. The ash samples were solubilized using nitric acid (10 %, wt/v). The solubilized samples obtained were analyzed for Na⁺, K⁺, Mg²⁺, Ca²⁺ using an atomic absorption

spectrophotometer (Perkin Elmer Ltd, 1982.), Cl^- (ion selective electrode, Orion), PO_4^{2-} (Sigma Diagnostics, Procedure # 670) and S (ICP, Cumber Land Valley Analytical Inc.).

The SID was calculated using the following equation:

$$[\text{SID}] = [\text{Na}^+ + \text{Ca}^{2+} + \text{K}^+ + \text{Mg}^{2+}] - [\text{Cl}^- + \text{PO}_4^{2-} + \text{S}^-]$$

where concentrations of individual elements are expressed as *mEq* /100 g microbial pellet.

Statistical Analysis

The experimental design was a randomized block design with a 2 x 3 x 3 x 2 x 2 factorial arrangement of treatments replicated four times. The replications were across runs with each run being one replicate. The treatments in the model were pellet type (LAB vs. SAB), buffer concentration (0.5x, 1x, 2x), buffer pH (5.8 vs. 6.8), feed (alfalfa hay vs. corn grain), and length of incubation (4, 14, or 24 h).

The statistical model used in the experiment was:

Mineral (g/100 g of microbial pellet) or SID (*mEq*/100 g of microbial pellet) = overall mean + pellet type + buffer treatment + buffer pH + feed treatment + length of incubation (h) + all interactions + run (random) + error

Depending on the significance of interactions (cut off at $P < 0.05$), linear and quadratic contrasts for concentration were evaluated separately for each pellet type (LAB, SAB), or for each combination of pellet type, buffer treatment, feed treatment, length of incubation and buffer pH. The data were tested for homogeneity of variance and effects were declared significant at ($P < 0.05$).

Results

Difference between solid and liquid associated bacterial fractions

Mineral composition of microbes was different between pellet types (Table 3.2). Ash content was lower for SAB than LAB ($P < 0.001$) but SID tended to be higher for SAB than LAB because of a decrease ($P < 0.001$) in mineral concentration of Na^+ , K^+ , Cl^- , Ca^{2+} , Mg^{2+} , PO_4^{2-} , and S^- .

Effect of Buffer Strength on Mineral Composition of Microbes

Mineral composition of microbes varied with concentration of buffer medium (Table 3.3). Increase in buffer strength increased ash content ($P < 0.001$) and tended to increase SID as buffer strength was associated with an increase in both the cation Na^+ ($P < 0.001$), and anions, Cl^- ($P < 0.001$) and PO_4^{2-} ($P = 0.01$) while, Mg was higher ($P < 0.001$) for 2x than for 0.5x and 1x buffer strength. However, the concentration of Ca^{2+} was lower for the 2x buffer than 0.5x buffer treatment ($P < 0.05$). Concentrations of K^+ and S^- were not affected by buffer strength.

There was a pellet type by buffer strength interaction for Na^+ (Figure 3.1 a). Sodium ion concentration increased more with increasing buffer strength for LAB than SAB fraction. There was a pellet type by buffer strength by length of incubation interaction for Cl^- concentration in the microbial pool (Figure 3.2 a). Chloride ion concentration was higher at 0.5x ($P < 0.01$) as compared to 1x and 2x buffer concentration for LAB fraction but for SAB fraction buffer strength did not affect the Cl^- concentration in the microbial pool.

There was a significant pellet type by buffer strength by feed type interaction for Ca^{2+} (Figure 3.3 a). The Ca^{2+} concentration for alfalfa hay decreased in the LAB fraction at 2x as compared to 0.5x and 1x (linear $P = 0.01$) whereas for corn grain it tended to increase at 2x buffer treatment (linear $P = 0.08$). Calcium ion concentration was not different between the two buffer strengths for SAB fraction. Significant pellet type by buffer strength by feed interaction was present for Mg^{2+} (Figure 3.4 a). The buffer strength did not affect Mg^{2+} concentration in the microbial pellet when incubated with alfalfa hay or corn grain for SAB fraction. While Mg^{2+} concentration was higher at 2x buffer strength as compared to 0.5x and 1x (linear $P < 0.001$, quadratic $P = 0.18$) when incubated with alfalfa hay for LAB fraction. Buffer concentration did not affect Mg^{2+} concentration in microbial pellet when incubated with corn grain for LAB fraction.

There was a pellet by buffer interaction for PO_4^{2-} (Figure 3.5 a). Inorganic phosphorus concentration in the microbial pool increased with increasing concentration of buffer ($P < 0.001$) for the LAB fraction, while for SAB fraction it tended to increase with increasing concentration of buffer ($P = 0.057$). Microbial SID concentration was higher for LAB than SAB (linear $P = 0.02$; quadratic $P = 0.2$) at increasing buffer strength (Figure 3.6 a).

Effect of pH on Mineral Composition of Microbes

The mineral concentration in the microbes varied with the change in pH of the buffer medium (Table 3.4). Low buffer pH decreased ash and SID by increasing Cl^- ($P < 0.001$), PO_4^{2-} ($P < 0.01$), and decreasing Na^+ ($P = 0.07$), Ca^{2+} ($P < 0.01$) and S^-

($P < 0.01$). The pH of the incubating medium did not affect K^+ and Mg^{2+} concentrations.

Sodium ion concentration was higher at pH 6.8 for LAB as compared to pH 5.8 ($P < 0.001$) but did not vary between buffer pH for SAB fraction (Figure 3.1 b). Chloride ion concentration was higher ($P < 0.001$) at pH 5.8 as compared to pH 6.8 at 14 h of incubation (Figure 3.2 b), other than that Cl^- concentration was not affected by pH of the medium. Calcium ion concentration was higher at pH 6.8 ($P < 0.001$) as compared to pH 5.8 in alfalfa hay for LAB fraction. The pH of the incubating medium had no other effect on Ca^{2+} concentration in the microbial pool (Figure 3.3 b). Concentration of Mg^{2+} was higher at pH 6.8 for LAB ($P < 0.05$) as compared to pH 5.8 in LAB fraction, whereas no effect of pH of the incubating medium was seen for SAB fraction (Figure 3.4 b). Inorganic phosphorus concentration decreased at pH 5.8 ($P = 0.07$) as compared to pH 6.8 for the LAB and SAB fraction (Figure 3.5 b). There was no effect of pH on SID concentration for the SAB fraction, whereas the SID for the LAB fraction decreased ($P < 0.001$) with decrease in pH (Figure 3.6 b).

Effect of Feed Type on Mineral Composition of Microbes

The mineral composition of microbes varied with the type of feed (Table 3.5). Ash content was higher ($P < 0.001$) and SID tended to be higher ($P = 0.067$) for alfalfa hay than corn grain because of an associated increase in K^+ ($P < 0.001$), Cl^- ($P = 0.057$), Ca^{2+} ($P < 0.001$), Mg^{2+} ($P = 0.04$), and S^{2-} ($P < 0.001$) concentrations in alfalfa hay than corn grain. The feed treatment did not affect the Na^+ and PO_4^{2-} concentrations of the microbial pool.

Effect of Length of Incubation on Mineral Composition of Microbes

Mineral concentration in microbes varied with the length of incubation (Table 3.6). Over the length of incubation, ash content decreased while SID remained unchanged as Na^+ ($P = 0.01$), K^+ ($P < 0.001$), Cl^- ($P < 0.01$) and PO_4^{2-} ($P < 0.04$) decreased from 4 to 24 h of incubation. Sodium ion concentration of microbial pool decreased at 14 h ($P < 0.01$) and tended to decrease at 24 h ($P = 0.062$), as compared to 4 h. Potassium ion concentration was lower at 14 h ($P < 0.01$) and 24 h ($P < 0.001$) as compared to 4 h of incubation but did not differ between 14 and 24 h of incubation. Chloride ion concentration decreased at 14 h ($P < 0.001$) and 24 h ($P < 0.01$) as compared to the concentration at 4 h. Calcium ion concentration in microbes decreased at 14 h ($P = 0.024$) as compared to 4 h. The length of incubation did not affect Mg^{2+} concentration in the microbial pool. Inorganic phosphorus concentration was decreased at 24 h ($P = 0.046$) as compared to 4 h of incubation. Sulfide ion concentration was lower at 24 h as compared to 4 h of incubation ($P < 0.01$), but was not different between 4 and 14 h of incubation.

Significant pellet by time by feed interaction was seen for K^+ (Figure 3.7) in the microbial pool. Potassium ion concentration in case of alfalfa hay increased at 14 h ($P = 0.001$) and 24 h ($P < 0.001$) as compared to 4 h for the SAB fraction. Concentration of K^+ in case of corn grain decreased at 24 h ($P = 0.01$) as compared to 14 h, for the SAB fraction. Potassium ion concentration in case of alfalfa hay decreased at 14 h ($P < 0.001$) and 24 h ($P < 0.001$) as compared to 4 h for LAB fraction. Concentration of K^+ in case of corn grain tended to decrease at 24 h ($P =$

0.09) as compared to 14 h, and at 14 h ($P < 0.001$) and 24 h ($P < 0.001$) as compared to 4 h for LAB fraction. Significant pellet type by incubation time by feed interaction was seen for S^- (Figure 3.8) in the microbial pool. Sulfide ion concentration decreased at 14 h ($P < 0.01$), and 24 h ($P = 0.031$, quadratic $P = 0.045$) in case of alfalfa hay, and at 14 h ($P < 0.001$) and 24 h ($P < 0.001$, quadratic $P = 0.012$) in case of corn grain for the SAB fraction. Sulfide ion concentration increased when alfalfa hay was incubated at 14 h ($P = 0.005$) and 24 h ($P = 0.1$, quadratic $P = 0.019$), and when corn grain was incubated at 24 h ($P = 0.017$) as compared to 4 h for the LAB fraction.

Discussion

Large numbers of studies have attempted to evaluate the chemical composition of liquid and solid associated fractions of the microbial pool and the factors that affect these differences. Differences have been shown in the chemical composition of LAB and SAB fraction of microbial pool (Czerkawski, 1977). Perez et al. (1998) showed that the rumen outflow of LAB and SAB are significantly affected by dietary and rumen environment. Thus, for prediction of rumen SID we need to know the microbial mineral composition as well as SID concentration as affected by contrasting treatments.

Results obtained in this study for the mineral composition are coherent with previous findings (Merry and McAllan 1983; Perez et al., 1998; Legay-Carmier and Bauchart, 1989) that differences exist between the LAB and SAB fractions. Ash content in this study was lower for SAB fraction as compared to LAB fraction, as shown by other researchers (Merry and McAllan, 1983). Published values of ash have been highly variable, varying from 5.0 g/100 g microbial pellet (Hoogenraad

and Hird, 1970) to almost 30.0 g/100 g of microbial matter (Smith and McAllan, 1973). Liquid associated bacteria have a higher mineral concentration compared to the SAB fraction, as LAB fraction is present in the mineral rich environment of the rumen fluid (Lantham, 1980). Microbial LAB fraction plays a lesser role in feed digestion and subsists on soluble feed components in the rumen fluid (Lantham, 1980). Thus, the LAB fraction is likely to incorporate more minerals in the cell as compared to SAB fraction which is associated with feed particles.

In the current study, the strength of the buffer medium affected the mineral composition of microbial pool. Ash content decreased but SID was not affected because of an increased uptake of Na^+ , Cl^- , and PO_4^{2-} . Potassium and S^- concentrations were not affected by increasing buffer strength. The most predominant rumen bacteria seem to contain enzymes whose activity requires K^+ (Caldwell et al., 1973). In our study, we found Na^+ and K^+ concentrations ranging from 2.34 to 4.13 g/100 g and 1.33 to 1.46 g/100 g microbial pellet on DM basis respectively. Martinez (1972) found 21 g K^+ per kg and 36 g Na^+ per kg DM in rumen bacterial sediment. The Na^+ concentration in our experiment agrees with the values reported by Martinez (1972) while, K^+ concentration was lower in our study, which may be explained by an electrochemical gradient across microbial membranes as Na^+ and K^+ , both being univalent can replace each other to maintain intracellular osmolality (Durand and Kawashima, 1980). Moreover, some rumen bacteria have been shown to be slightly halophilic (Caldwell et al., 1973) which might explain the high Na^+ content in the microbes. Furthermore, it has been shown that a relatively large quantity of Na^+ is

obligatory for all predominant rumen *Bacteriodes* species (Caldwell & Arcand, 1974) as well as for other genera of rumen bacteria (Bennink et al., 1978).

Chloride ion acts as a strong anion source in the rumen (Kohn, 2000); thus, in the microbial cytoplasm it may play a role in decreasing the intracellular pH. Chloride ion content may be dependent on Na^+ transport, to maintain intracellular pH of the microbial cells.

Calcium ion functions at the cellular membrane or external to the membrane in microbes (Martinez, 1972). In our study, the calcium ion concentration ranged from 0.24 to 0.3 %, and decreased with increase in the concentration of buffer. These values agree with previous reported values of 0.1 to 0.3 g/kg of bacilli (Durand & Kawashima, 1980). Magnesium ion is required for many intra-cellular processes (Weinberg, 1977), most of which are associated with ribosomes. We found the magnesium ion concentration to increase with increasing buffer concentration in the microbial pool. This is in consistence with the increased binding of Mg^{2+} to cell wall in medium of higher concentration of buffer (Durand & Kawashima, 1980). On dry weight basis total P content of rumen microorganisms range from 2 to 6 % (Kaufmann, 1976). The mean PO_4^{2-} concentration in this study ranged from 1.1g/100 g to 2.48g/100 g microbial pellet on DM basis. However, there was an increase in rumen microbial PO_4^{2-} concentration with increasing concentration of buffer, suggesting that 2x buffer treatments may increase the microbial growth.

Low buffer pH decreased ash and SID concentration due to an increase in Cl^- , and PO_4^{2-} and a decrease in Na^+ , Mg^{2+} and Ca^{2+} in the microbial pool. SID of microbial pool decreased as the pH of the medium decreased due to an increase in

anions and a decrease in cations from the microbial pool. Decrease in Na^+ and Ca^{2+} concentrations with decrease in pH can be attributed to Na^+/H^+ and $\text{Ca}^{2+}/2\text{H}^+$ anti-porters (Hughes and Poole, 1989) which causes Na^+ and Ca^{2+} to be expelled out from the microbial pool. Moreover, a decrease in pH of the media may favor an increase in growth of microbes that can resist low rumen pH. These microbes may have higher intra-cellular concentrations of Cl^- and PO_4^{2-} to resist changes in the outside environment.

The alfalfa hay treatment had higher ash content as compared to corn treatment. This result is in agreement with previous studies that showed higher OM content for high concentrate diets as compared to high forage diet (Perez et al., 1996; 1998). In the current study, ash content increased but SID tended to increase for alfalfa hay as compared to corn grain because K^+ , Cl^- , Mg^{2+} , Ca^{2+} and S^- concentration were all higher for the former. Sodium ion concentration in the buffer medium was very high, and this may account for most of the variation in the microbial pool; hence, feed treatment had little effect on Na^+ concentration. Higher K^+ , Ca^{2+} , Mg^{2+} and S^- content of alfalfa hay as compared to corn grain can account for higher concentration of these minerals in the microbes. Calcium and Mg ion concentrations in the microbial pool depend upon the type of diet (Durand and Kawashima, 1980). In this study, we used two contrasting diets and thus, concentration of these minerals was different for microbes. Alfalfa hay has more S^- content, due to higher concentration of protein and the presence of preformed sulfur containing amino acids (McMeniman, 1976), which may affect the level of incorporation of sulfur into the microbial pool.

Ash content in the microbial pellet decreased over the length of incubation but SID was unaffected as concentrations of Na^+ , K^+ , Cl^- , Ca^{2+} , PO_4^{2-} and S^- decreased with increase in length of incubation. Increase in uptake of nutrients by microorganisms as they grow may decrease the ash content of microbes with time. Sodium ion concentration was highest for incubation at 4 h, as the microbes were growing rapidly and medium was highly saturated with Na^+ . However, as time of incubation increases, bacteria mature and this may have lead to a decrease in uptake of Na^+ into the microbial cytoplasm. Potassium ion concentration for incubation at 4 h was different from 14h and 24 h. At 4 h, microbes are growing but at 14 and 24 h, microbes are reaching maturity; thus mineral uptake by microbes may have slowed. Concentration of Cl^- may be regulated by the concentration of univalent cations (i.e. Na^+ and K^+) to maintain the intra-cellular pH. Phosphate ion content decreased with length of incubation. As the cells are actively growing, there is increased protein synthesis and this increased the PO_4^{2-} content of microbes at 14h and 24 h. Decrease in S^- ion concentration in the microbial pool may also be associated with a decrease in protein synthesis as the microbes mature.

Implications

This research studies factors affecting microbial mineral content and SID in vitro; the reported values can be used to predict changes in rumen SID and pH as affected by diet, osmolarity of medium, length of incubation and pH of the medium. The differences in mineral composition of LAB and SAB fractions of the microbial pool, type of feed offered and diurnal variation show the importance of defining a representative microbial fraction. On average, microbes took up more cations than

anions from the media. Calculated SID of the microbial pool decreased with pH but was not affected by other treatments due to a similar change in uptake of cations and anions. The results enable us to predict change in SID due to microbial growth which could further affect rumen pH.

Table 3.1. Composition of buffers

Buffer:	1x, pH 6.8 ^{ab}	1x, pH 5.8 ^{ac}	2x, pH6.8 ^{ad}	0.5x, pH6.8 ^{ae}
Deionized water (L)	1.0	1.0	1.0	1.0
(NH ₄)HCO ₃ (g)	4.0	4.0	8.0	2.0
NaHCO ₃ (g)	35.0	35.0	70.0	17.5
Macro-mineral solution				
Deionized water (L)	1.0	1.0	1.0	1.0
Na ₂ HPO ₄ (g)	5.7	5.7	11.4	2.83
KH ₂ PO ₄ (g)	6.2	6.2	12.4	3.1
MgSO ₄ ·7H ₂ O	0.6	0.6	1.2	0.3
Micro-mineral solution				
Deionized water (mL)	100.0	100.0	100.0	100.0
CaCl ₂ ·2H ₂ O (g)	13.2	13.2	26.4	6.6
MnCl ₂ ·4H ₂ O(g)	10.0	10.0	20.0	5.0
CoCl ₂ ·6H ₂ O(g)	1.0	1.0	2.0	0.5
FeCl ₃ ·6H ₂ O (g)	8.0	8.0	16.0	4.0

^aResazurin solution 0.1% (wt/vol) was added to all the buffers.

^bConcentration of buffer at 1 times buffer concentration at pH 6.8.

^cConcentration of buffer at 1 times buffer concentration at pH 5.8.

^dConcentration of buffer at 2 times buffer concentration at pH 6.8.

^eConcentration of buffer at 0.5 times buffer concentration at pH 6.8.

Table 3.2. Difference in mineral composition of pellet type (LAB vs SAB)^a

Item	LAB	SAB	SEM
Ash ^b	15.37 ^e	10.86 ^f	0.42
SID ^c	114.28	120.24	10.79
Minerals ^d			
Sodium ion	157.83 ^e	116.52 ^f	7.39
Potassium ion	40.43 ^e	32.75 ^f	3.33
Chloride ion	53.31 ^e	8.46 ^f	2.54
Calcium ion	14.47 ^e	10.48 ^f	2.49
Magnesium ion	22.22 ^e	17.28 ^f	2.47
Phosphate ion	59.75 ^e	42.03 ^f	1.27
Sulfide ion	8.13 ^d	6.25 ^e	0.31

^aLiquid and solid associated bacteria^bAsh g/100 g of microbial pellet.^cSID *mEq*/100 g is *mEq* of SID in 100 grams of microbial pellet on dry matter basis, calculated as $[\text{Na}^+ + \text{Ca}^{2+} + \text{K}^+ + \text{Mg}^{2+}] - [\text{Cl}^- + \text{PO}_4^{2-} + \text{S}^-]$.^dMineral *meq*/100 g is milli-equivalent of mineral in 100 g of microbial pellet on dry matter basis^{e,f}Mean in same row without a common superscript differ, ($P < 0.05$).**Table 3.3.** Effect of buffer treatment on minerals composition of microbes

Item	0.5x, pH6.8 ^a	1x, pH6.8 ^b	2x, pH6.8 ^c	SEM
Ash ^d	11.99 ^g	13.36 ^h	15.31 ⁱ	0.60
SID ^e	103.81 ^g	137.15 ^h	149.06 ^g	12.85
Minerals ^f				
Sodium ion	101.74 ^g	147.39 ^h	179.56 ⁱ	10.43
Potassium ion	34.03	37.10	37.34	3.58
Chloride ion	12.96 ^g	19.75 ^g	34.98 ^h	3.95
Calcium ion	14.97 ^g	13.97 ^{gh}	11.97 ^h	2.54
Magnesium ion	18.93 ^g	18.11 ^g	26.33 ^h	2.55
Phosphate ion	44.56 ^g	25.57 ^h	62.78 ⁱ	1.65
Sulfide ion	7.81	8.44	8.13	0.41

^aConcentration of buffer at one half normal buffer strength at pH 6.8.^bConcentration of buffer at normal buffer strength at pH 6.8.^cConcentration of buffer at twice normal buffer strength at pH 6.8.^dAsh g/100 g of microbial pellet.^eSID *mEq*/100 g is *mEq* of SID in 100 grams of microbial pellet on dry matter basis, calculated as $[\text{Na}^+ + \text{Ca}^{2+} + \text{K}^+ + \text{Mg}^{2+}] - [\text{Cl}^- + \text{PO}_4^{2-} + \text{S}^-]$.^fMineral *meq*/100 g is milli-equivalent of mineral ion in 100 g of microbial pellet on dry matter basis^{g,h,i}Mean in same row without a common superscript differ, ($P < 0.05$).

Table 3.4. Effect of pH on mineral composition of microbes

Item	1x, pH 6.8 ^a	1x, pH 5.8 ^b	SEM
Ash ^c	13.36 ^f	11.80 ^g	0.60
SID ^d	137.15 ^f	79.01 ^g	12.85
Minerals ^e			
Sodium ion	147.39 ^f	120.0 ^g	10.43
Potassium ion	37.10	38.13	3.58
Chloride	19.75 ^f	55.57 ^g	3.95
Calcium ion	13.97 ^f	9.48 ^g	2.49
Magnesium ion	18.11	16.46	2.46
Phosphate ion	25.57 ^f	45.32 ^g	1.77
Sulfide ion	8.44 ^f	3.75 ^g	0.31

^aConcentration of buffer at normal buffer strength at pH 6.8.

^bConcentration of buffer at normal buffer strength at pH 5.8.

^cAsh g/100 g of microbial pellet.

^dSID *mEq*/100 g is *mEq* of SID in 100 grams of microbial pellet on dry matter basis, calculated as $[\text{Na}^+ + \text{Ca}^{2+} + \text{K}^+ + \text{Mg}^{2+}] - [\text{Cl}^- + \text{PO}_4^{2-} + \text{S}^-]$.

^eMineral *mEq*/100 g is milli-equivalent of mineral ion in 100 g of microbial pellet on dry matter basis

^{f,g}Mean in same row without a common superscript differ ($P < 0.05$).

Table 3.5. Effect of feed treatment on minerals composition of microbes

Item	Alfalfa hay ^a	Corn grain ^b	SEM
Ash ^c	13.95 ^f	12.28 ^g	0.42
SID ^d	129.62	104.91	10.79
Minerals ^e			
Sodium ion	143.48	130.87	7.39
Potassium ion	41.71 ^f	31.22 ^g	1.28
Chloride	34.41	27.36	2.82
Calcium ion	17.96 ^f	6.99 ^g	2.49
Magnesium ion	21.40 ^f	18.11 ^g	2.47
Phosphate ion	51.39	50.38	1.27
Sulfide ion	9.06 ^f	5.00 ^g	0.31

^aalfalfa hay

^bcorn grain

^cAsh g/100 g of microbial pellet.

^dSID *mEq*/100 g is *mEq* of SID in 100 grams of microbial pellet on dry matter basis, calculated as $[\text{Na}^+ + \text{Ca}^{2+} + \text{K}^+ + \text{Mg}^{2+}] - [\text{Cl}^- + \text{PO}_4^{2-} + \text{S}^-]$.

^eMineral *mEq*/100 g is milli-equivalent of mineral ion in 100 g of microbial pellet on dry matter basis

^{f,g}Mean in same row without a common superscript differ ($P < 0.05$).

Table 3.6. Effect of length of incubation on mineral composition of microbes:

Item	4 h ^a	14h ^b	24h ^c	SEM
Ash ^d	15.19 ^g	12.14 ^h	12.02 ^h	0.52
SID ^e	128.11	105.92	117.76	11.87
Minerals ^f				
Sodium ion	156.95 ^g	121.30 ^h	133.48 ^h	9.13
Potassium ion	40.17 ^g	36.08 ^h	33.52 ^h	3.33
Chloride	41.75 ^g	25.70 ^h	25.11 ^h	3.38
Calcium ion	13.97 ^g	10.47 ^h	12.97 ^{gh}	2.49
Magnesium ion	20.58	20.58	18.11	2.47
Phosphate ion	54.18 ^g	50.38 ^h	48.10 ^h	1.52
Sulfide ion	7.50 ^g	7.19 ^{gh}	6.56 ^g	0.31

^a4h of incubation.^b14h of incubation.^c24h of incubation.^d Ash g/100 g of microbial pellet.^eSID *mEq*/100 g is *mEq* of SID in 100 grams of microbial pellet on dry matter basis, calculated as $[\text{Na}^+ + \text{Ca}^{2+} + \text{K}^+ + \text{Mg}^{2+}] - [\text{Cl}^- + \text{PO}_4^{2-} + \text{S}^-]$.^fMineral *meq*/100 g is milli-equivalent of mineral ion in 100 g of microbial pellet on dry matter basis.^{g,h}Mean in same row without a common superscript differ ($P < 0.05$).

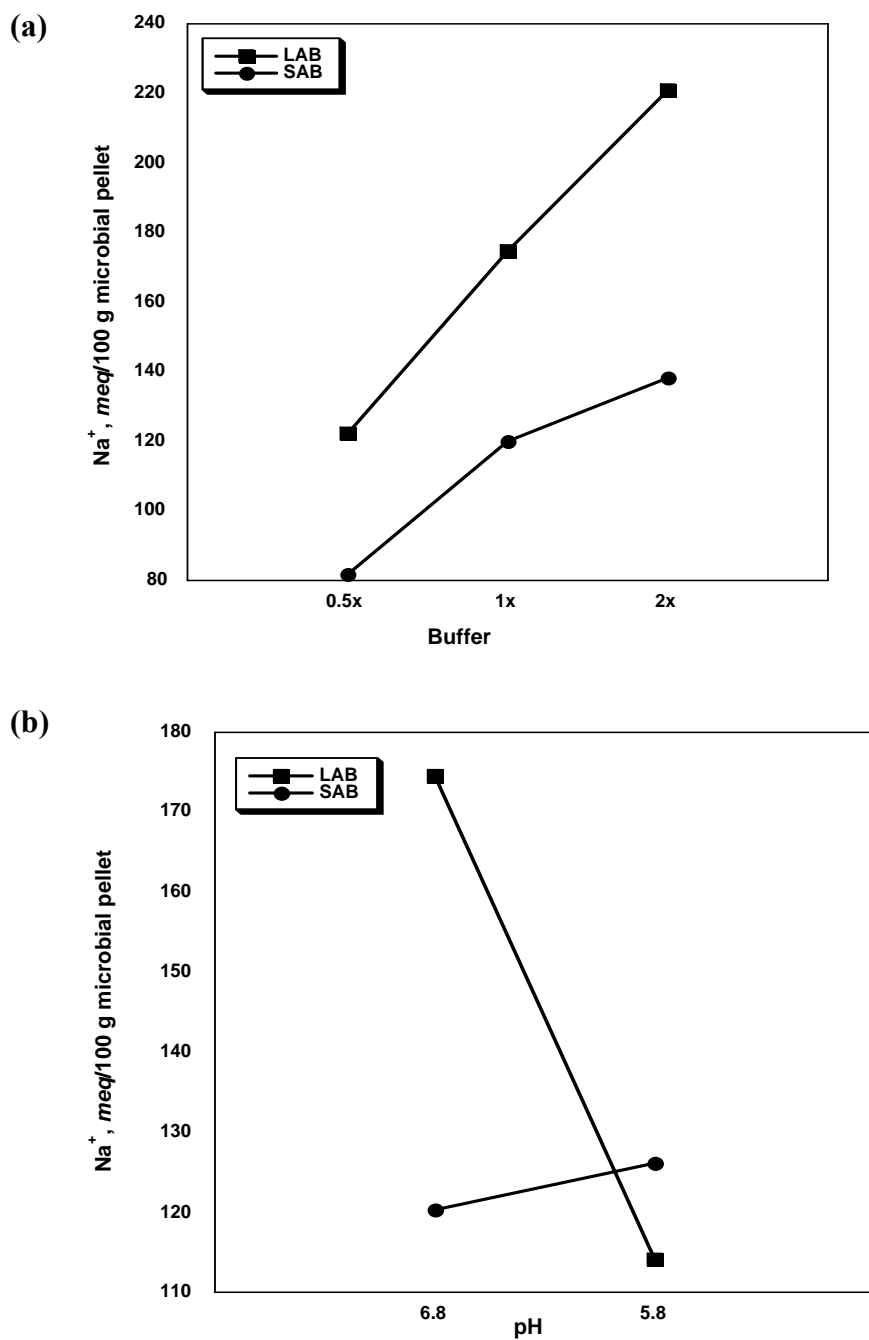


Figure 3.1. Effect of (a) pellet type by buffer strength ($P < 0.01$) and (b) pellet type by pH ($P < 0.01$) on Na^+ concentration in microbial pellet. Pellet types are liquid associated bacteria (LAB), and solid associated bacteria (SAB). Buffer is at 0.5 times normal concentration (0.5x), normal concentration (1x), and twice normal concentration (2x). pH is 5.8 and 6.8 at normal buffer concentration.

(a)

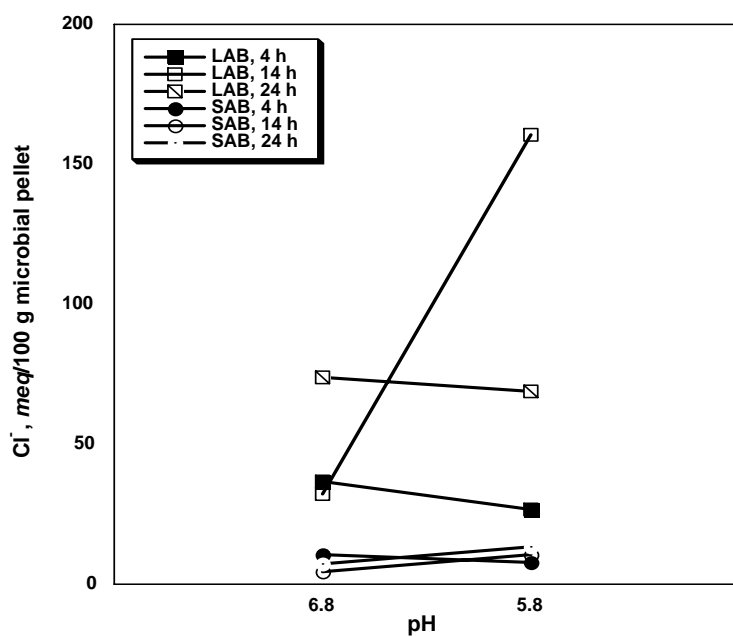
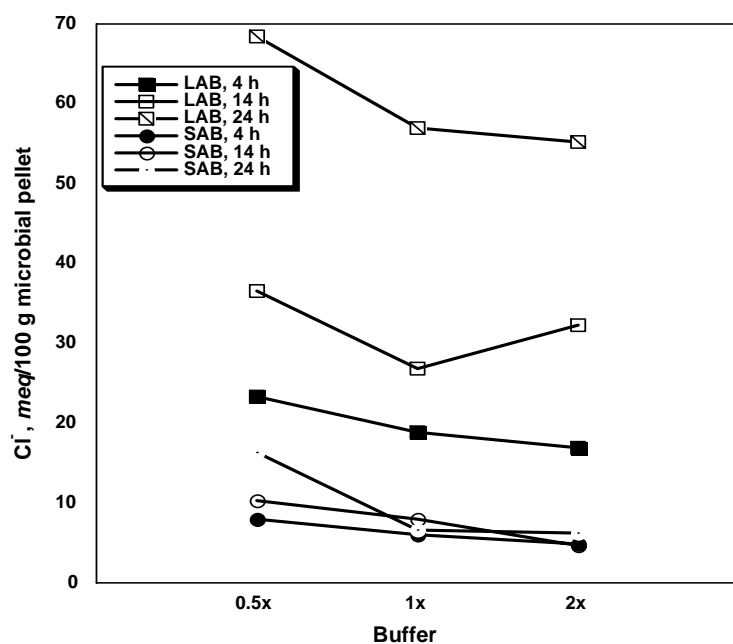


Figure 3.2 Effect of (a) pellet type by buffer strength by length of incubation and (b) pellet type by pH by length of incubation on Cl⁻ concentration in microbial pellet. Pellet types are liquid associated bacteria (LAB), and solid associated bacteria (SAB). Buffer concentration is at 0.5 times (0.5x), normal (1x), and twice (2x) normal concentrations. Length of incubation is 4 hours, 14 hours, and 24 hours of incubation.

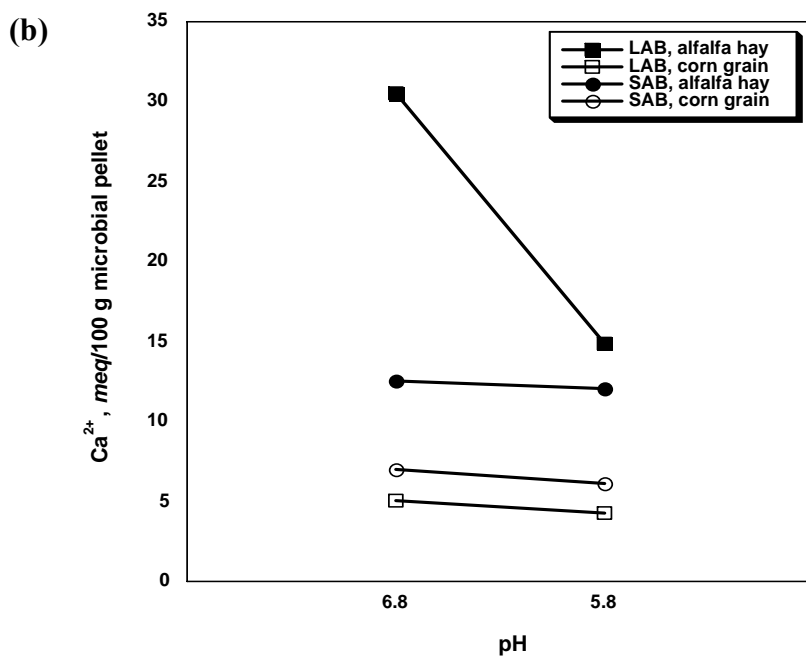
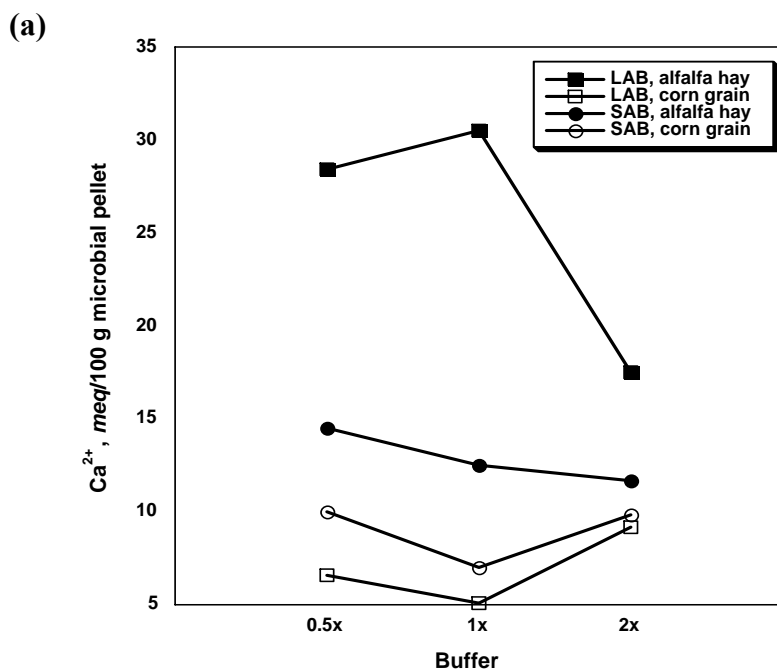
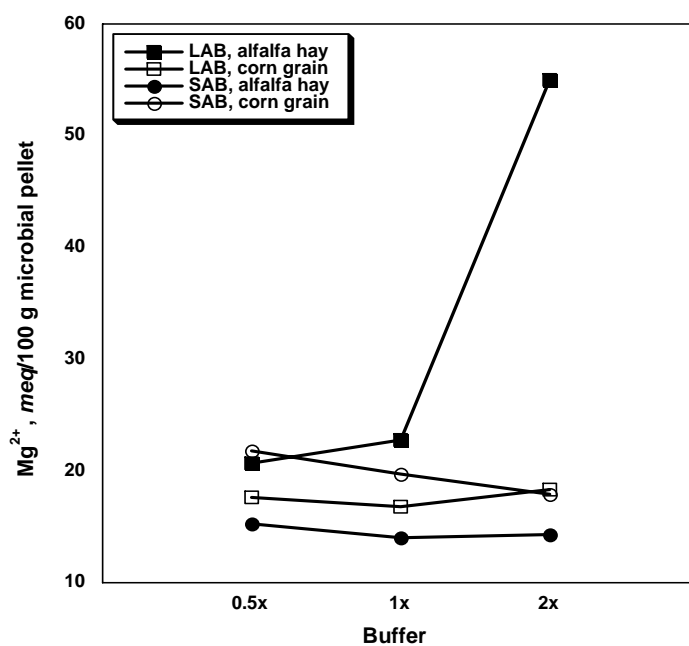


Figure 3.3. Effect of (a) pellet type by buffer strength by feed, and (b) pellet type by pH by feed on Ca^{2+} concentration in microbial pellet. Pellet types are liquid associated bacteria (LAB), solid associated bacteria (SAB). Buffer is at 0.5 times (0.5x), normal (1x), and twice (2x) the normal concentration. Feed treatments are alfalfa hay and corn. pH is 5.8 and 6.8 at normal (1x) buffer concentration.

(a)



(b)

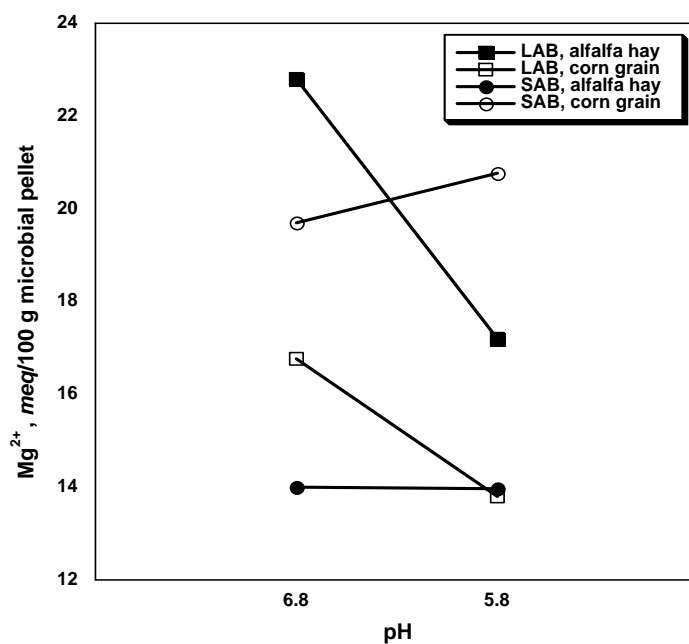
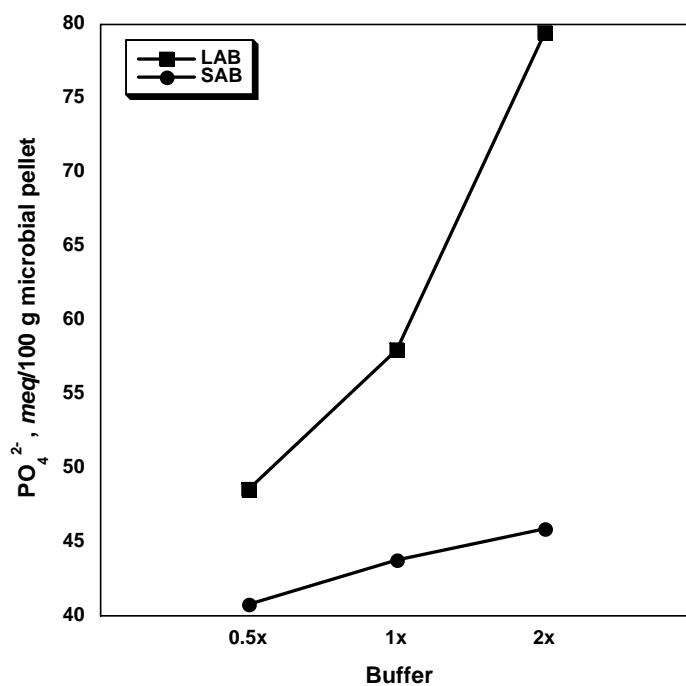


Figure 3.4. Effect of (a) pellet type by buffer strength by feed, and (b) pellet type by pH by feed, on Mg^{2+} concentration in microbial pellet. Pellet types are liquid associated bacteria (LAB), solid associated bacteria (SAB). Buffer is at 0.5 times on (0.5x), normal (1x), and twice (2x) the normal concentration. Feed treatments are alfalfa hay and corn. pH is 5.8 and 6.8 at normal (1x) buffer concentration.

(a)



(b)

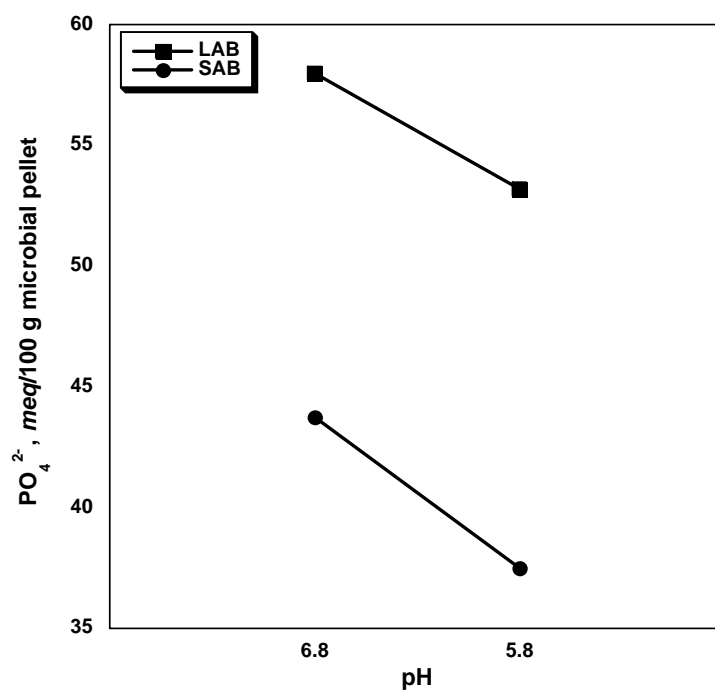
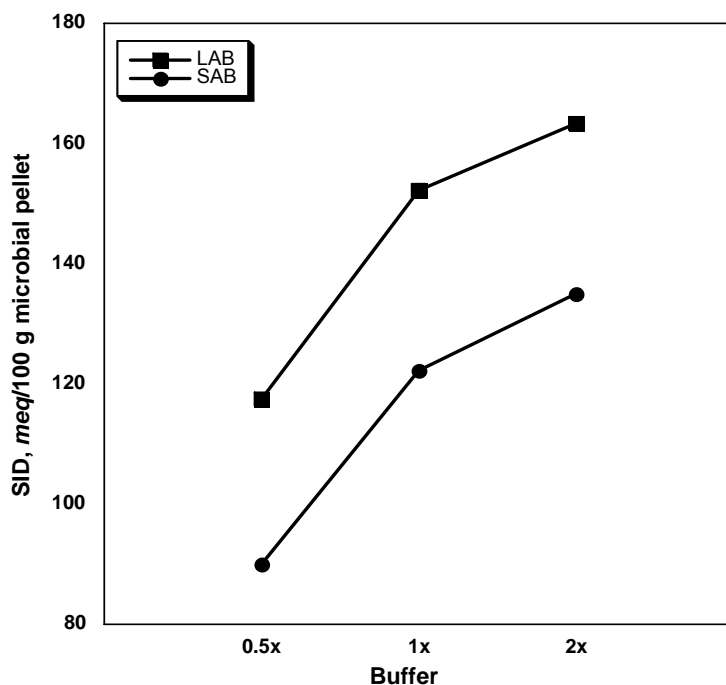


Figure 3.5. Effect of (a) pellet type by buffer strength, and (b) pellet type by pH on PO_4^{2-} concentration in rumen microbial pool. Pellet types are liquid associated bacteria (LAB), and solid associated bacteria (SAB). Buffer is at 0.5 times (0.5x), normal (1x), and twice (2x) the normal concentration.

(a)



(b)

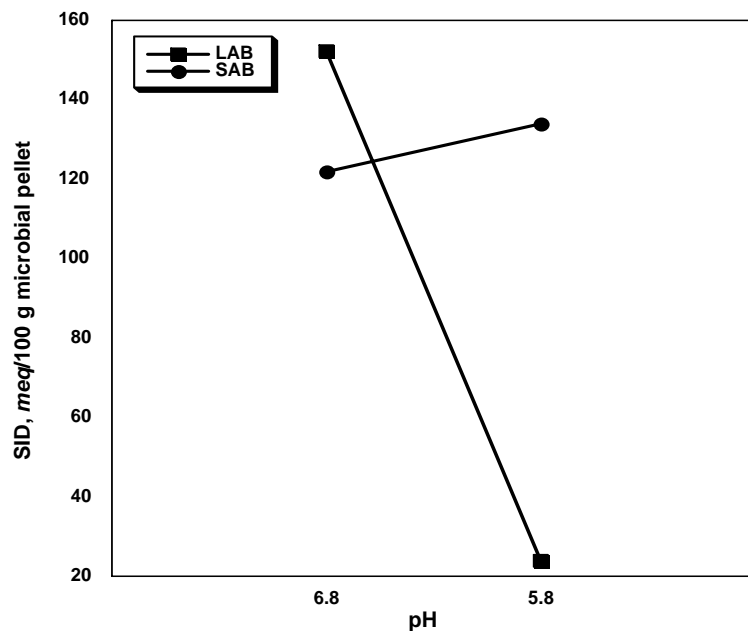


Figure 3.6. Effect of (a) pellet type by buffer strength, and (b) pellet type by buffer pH on SID $mM/100\text{ g}$ is mM of strong ion difference per 100 gram of microbial pellet per dry basis. Pellet types are liquid associated bacteria (LAB), and solid associated bacteria (SAB). pH is 5.8 and 6.8 at normal buffer concentration. Buffer is at 0.5 times (0.5x), normal (1x), and twice (2x) the normal concentration.

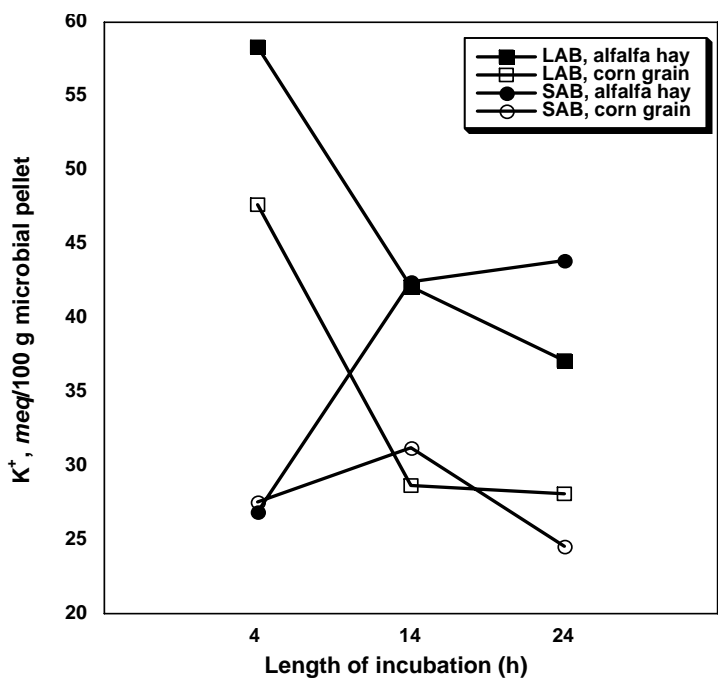


Figure 3.7. Effect of pellet type by incubation time by feed type on K^+ concentration in the microbial pellet. Pellet types are liquid associated bacteria (LAB), and solid associated bacteria (SAB). Length of incubation represents 4 hours, 14 hours, and 24 hours of incubation.

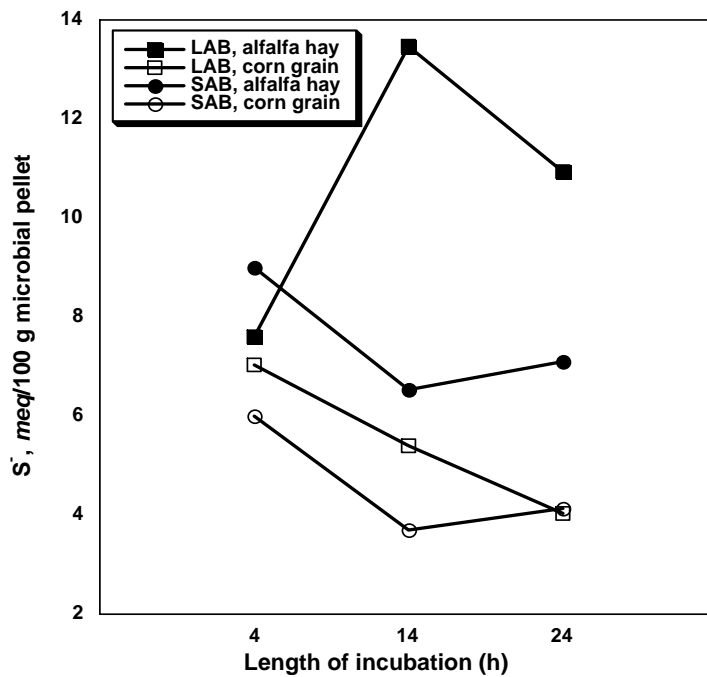


Figure 3.8. Effect of pellet type by length of incubation by feed treatment S⁻ concentration in microbial pellet. Pellet types are liquid associated bacteria (LAB), and solid associated bacteria (SAB). Length of incubation represents 4 hours, 14 hours, and 24 hours of incubation. Feed treatments are alfalfa hay and corn grain.

Appendix

Table 3.7 Significant effects in model and variance accounted for each treatment (%)

Item	Significant Effects ($P < 0.1$)	Pellet type ^a	Buffer treatment ^b	Feed treatment ^c	Length of incubation ^d
Ash ^e	Pellet type***; Buffer***; Feed*; Time ***	19.3	7.49	2.63	8.16
SID ^f	Pellet type [†] ; Buffer [†] ; Feed [†] ; P X B*	14.36	18.21	1.75	0.49
Mineral ^g					
Na ⁺	Pellet type***; Buffer***; Time*; P X B *	8.62	15.05	0.6	3.26
K ⁺	Pellet type***; Feed***; Time*; P X F [†] ; P X B [†] ; P X T***; F X T*; P X F X T*	16.13	2.07	14.1	14.5
Cl ⁻	Pellet type***; Buffer***; Feed [†] ; Time*; P X B***; P X T*; B X T*; P X B X T*	33.49	22.04	1.08	5.85
Ca ²⁺	Pellet type*; Buffer [†] ; Feed***; Time [†] ; P X F ***; P X B [†] ; F X T*; F X B*; P X F X T*	9.04	6.5	29.63	4.32
Mg ²⁺	Pellet type*; Buffer*; Feed*; P X F ***; P X B***; P X T [†] ; F X T*; P X F X T*	15.72	15.36	11.42	3.87
PO ₄ ²⁻	Pellet type***; Buffer***; Time*; P X B***	31.96	22.33	0.13	2.29
S ⁻	Pellet type***; Buffer [†] ; Feed***; P X F*; P X B***; P X T***; F X B*; F X T*; P X F X T*	13.56	25.82	23.87	5.97

^a% variance in the mineral composition explained by pellet type.

^b% variance in the mineral composition explained by buffer treatment with pH confounded with in buffer.

^c% variance in the mineral composition explained by feed treatment.

^d% variance in the mineral composition explained by length of incubation.

^e Ash g/100 g of microbial pellet.

^fSID *mEq*/100 g is *mEq* of strong ion difference per 100 gram of microbial pellet on dry matter basis, calculated as $[Na^{+} + Ca^{2+} + K^{+} + Mg^{2+}] - [Cl^{-} + PO_4^{2-} + S^{-}]$.

^gMineral *meq*/100 g is milli-equivalent of mineral ion per 100 gram of microbial pellet on dry matter basis.

***($P < 0.001$);*($P \leq 0.05$); [†]($P \leq 0.1$)

P X B= pellet type x Buffer type; P X F = pellet type x feed treatment; P X T= pellet type x time; P X F X T = pellet type x feed x time;

P X B X T= pellet type x feed x time

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